The combined effects of pCO_2 and nitrogen conditions on the growth and carbon acquisition of the freshwater cyanobacterium *Microcystis aeruginosa*.

Master Thesis

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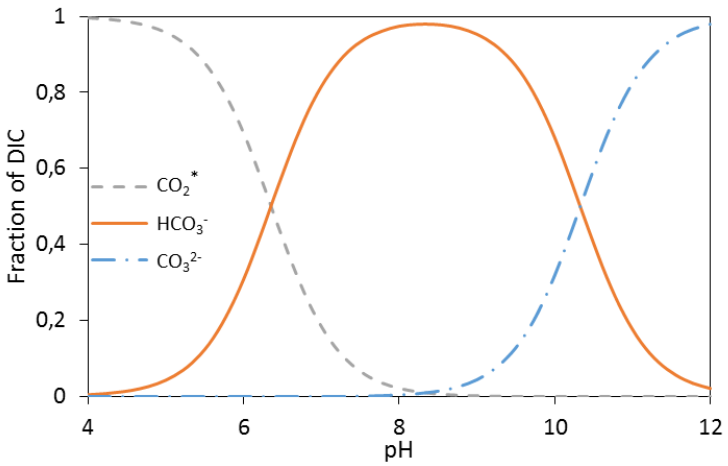
Abstract

Elevated CO_2 concentrations in freshwater ecosystems, driven by the anthropogenic increase of atmospheric pCO_2, may affect the growth, stoichiometry and carbon acquisition of phytoplankton. The direction and extent of these changes may be dependent on other environmental factors, such as nutrient loads. In this study we investigated the response of three strains of the freshwater cyanobacterium, *Microcystis aeruginosa*, to a wide range of pCO_2 under replete and deplete nitrogen conditions, in order to investigate their combined effects. This species is known in particular to cause intensive harmful blooms during warm periods in eutrophic systems. These blooms can deplete CO_2 concentrations to limiting conditions and may therefore be promoted by increased CO_2 availability. We found for all strains that increased pCO_2 affects C:N ratios and that the direction and extent of the response is dependent on the imposed nitrogen conditions. Specifically, C:N ratios increased with pCO_2 under nitrogen depleted conditions, while they decreased under nitrogen replete conditions. Furthermore, we found impaired growth rates at both low pCO_2 and at high pCO_2. This may be closely related to changes in carbon acquisition or putative photoinhibition. Changes in carbon acquisition are investigated using a single compartment model for stable carbon isotope fractionation (ε_p). *Microcystis* generally takes up bicarbonate as secondary carbon source. With ε_p calculations it is shown that cells become less reliant on bicarbonate at high pCO_2 and take up more CO_2 instead. The calculations also show that the total carbon uptake increases with increasing pCO_2 in nitrogen limited conditions. This is closely related to an increase in CO_2 leakage from cells at high pCO_2. These changes in carbon acquisition may have consequences for the growth and the energy balance of cells. Next to being a useful tool to track changes in carbon acquisition, the relation between pCO_2 and ε_p is also of particular interest for the reconstruction of past pCO_2. ε_p has been shown to increase with increasing pCO_2. This response interacts with the imposed nitrogen conditions, leading to a higher sensitivity to pCO_2 during nitrogen depleted than in nitrogen replete conditions. The observed strong relationship between ε_p and pCO_2 may support the development of CO_2 proxies, though this will be complicated by the interactive effect with nitrogen conditions. This study highlights the changes that occur at a physiological level in response to elevated pCO_2 under nitrogen replete/deplete conditions. The results show that elevated pCO_2 may promote *Microcystis* growth when it alleviates CO_2 limitation, but that it may impede growth when it becomes saturating. Large changes in C:N stoichiometry indicate a large flexibility of cyanobacteria. If the physiological changes observed in this study provide an ecological advantage for cyanobacteria it may have implications for the phytoplankton community composition. In all, the impacts of elevated pCO_2 on cyanobacterial blooms will strongly depend on the concurrent CO_2 and nitrogen concentrations, which may strongly influence the success of cyanobacteria in future freshwater ecosystems.

Introduction

Atmospheric CO_2 concentration has increased from 270 to 400 ppm since the beginning of the industrial revolution and could, according to the high emission scenario RCP8.5, reach ~950 ppm by the year 2100 (Collins *et al*. 2013). The increase in atmospheric CO_2 causes global warming and other climate change effects that may have large consequences for the aquatic environment. A major impact of increased atmospheric pCO_2 is the uptake of CO_2 by the oceans, which lowers the pH and shifts the equilibrium of carbonate species to more CO_2, more HCO_3^-, and less CO_3^{2-}, a process commonly referred to as ocean acidification (Wolf-Gladrow *et al*. 1999; Caldeira and Wickett, 2003; Doney *et al*. 2009). In contrast to the oceans, most freshwater lakes are supersaturated in pCO_2 (Cole *et al*. 1994). This is because the pCO_2 in lakes is mainly a result of metabolic processes, fueled by external organic material (Sobek *et al*. 2005). This organic material, mostly in the form of dissolved organic carbon (DOC), is internally recycled to pCO_2 and resupplied by allochthonous DOC from the terrestrial environment (Sobek *et al*. 2005). Climate change could affect the magnitude of the DOC flux into lakes. Depending on the local settings, an increase in the input of allochthonous DOC could occur due to climate effects as increased precipitation, longer growing seasons, increased soil temperatures and the thawing of permafrost (Hinton 1997, Adrian *et al*. 2009). Although there will be a strong variation in the response of lakes to a changing climate (Adrian *et al*. 2009), depending on the local settings, it is clear that rising atmospheric pCO_2 and increased DOC input potentially increases lake pCO_2. As a consequence, this increase in pCO_2 may affect freshwater phytoplankton.

pCO_2 of natural waters is strongly linked to the chemistry of the water. Freshwater systems contain a large number of weak acids and bases. These weak acids and bases play a major role in determining the pH of the water (Morel and Hering, 1993). In most natural waters the most important acid/base system is the carbonate system with total concentrations of about 1mM on average in freshwater systems (Morel and Hering, 1993). The main sources of carbonate in the water are dissolution of carbonate rock, atmospheric CO_2 and respiration (Morel and Hering, 1993). Unlike most other gases, CO_2 not only dissolves in water, but also reacts with water to form carbonic acid (H_2CO_3). Carbonic acid can dissociate in water to form free protons (H+) and the conjugate bases bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}). These dissociation reactions, listed below, occur very fast and in general it is safe to assume there is a thermodynamic equilibrium between the species (Sarmiento and Gruber, 2004). pH has a major role in determining the dominant carbonate species (fig. 1). This shows a large pH dependence of the distribution of the carbonate species. The species CO_2 (aq) and H_2CO_3 are difficult to distinguish analytically (Sarmiento and Gruber, 2004). Therefore they are commonly combined and expressed as CO_2*, a hypothetical species representing the sum of the species CO_2 (aq) and H_2CO_3 (Sarmiento and Gruber, 2004).



CO_2 (g) ⇔ CO_2 (aq)

CO_2 (aq) ⇔ H_2CO_3

H_2CO_3 ⇔ $H^+ + HCO_3^-$

HCO_3^- ⇔ $H^+ + CO_3^{2-}$

Figure 1. Carbonate system. *The contribution of carbonate species to DIC at 25°C are shown as function of pH. Next to the figure the relevant reactions are shown.*

An increase in pCO_2 may have strong implications for phytoplankton. Enhanced pCO_2, for instance, has been suggested to increase overall algal productivity (Schippers *et al*. 2004). However, there seems to be a strong variation in the response to increased pCO_2 among species (Schippers *et al*. 2004). While an increased availability of CO_2 may enhance growth, the concurrent decrease in pH may have negative consequences, especially for calcifying organisms which may have reduced rates of calcification (Riebesell *et al*. 2000a). The pCO_2 sensitivity seems to vary strongly between different phytoplankton species and even between strains (Langer *et al*. 2006. Langer *et al*. 2009). This sensitivity has been shown to depend on other resource conditions, such as light (e.g. Rokitta and Rost, 2012). An opposite trend in pH may occur when there are high levels of photosynthetic activity by phytoplankton, also called phytoplankton blooms, which typically occur in marine coastal waters and inland waters when nutrients are available in excess. Some species of phytoplankton are known to cause major ecological problems at high population densities and are therefore referred to as harmful algal blooms (HAB). A potential increase in the occurrence of HABs by climate change is therefore subject of ongoing research (HARRNESS, 2005). While the combination of interacting factors leading to HAB formation is not yet fully understood, some recent increases in HAB formation seem to be linked to increasing eutrophication and temperature (HARRNESS, 2005; Paerl and Huisman, 2008; Paul, 2008, Heisler *et al*. 2008; O'Neil *et al*. 2012).

An important group of phytoplankton causing harmful blooms in eutrophic systems are the cyanobacteria. Bloom forming cyanobacteria may produce neuro- cyto- and hepatotoxins that are hazardous to animal or human health (Sivonen 1996; Sivonen and Jones 1999; Paerl *et al*., 2001; HARRNESS, 2005). Dense blooms can even cause oxygen deprivation leading to fish kills. These blooms occur particularly in eutrophic systems during warm summers (Sivonen and Jones 1999). Many cyanobacteria can form intracellular gas vesicles that increases their buoyancy. This allows cyanobacteria to float near the surface and create dense surface blooms in stratified waters (Huisman *et al*. 2004; Visser *et al*. 2016). These surface blooms are effective in outcompeting other phytoplankton, leading to blooms that may be maintained for weeks to months. A possible explanation for the effective suppression of other phytoplankton during blooms is the shading effect surface cyanobacteria have on nonbuoyant phytoplankton (Shapiro 1973; Klemer 1989; Pearl and

Huisman 2008). A few effects of climate change have been considered to benefit the formation of harmful cyanobacteria blooms over other phytoplankton growth. Beyond the direct effect of higher temperatures that are considered to be beneficial to the maximum specific growth rate of cyanobacteria, possibly leading to increased resistance to grazing (Jöhnk *et al*. 2008; Lürling *et al.* 2013), an important indirect effect results from stratification, which is driven by temperature. Increased temperatures may strengthen stratification or lengthen the period lakes are stratified, providing longer optimal conditions for cyanobacteria blooms (Pearl and Huisman 2008; O'Neil et al 2012; Lürling et al. 2013; Visser *et al*. 2016). Another factor that may affect cyanobacteria blooms is the effect of CO_2. Due to the high cell densities in cyanobacteria blooms, nutrients rapidly decline and the cells may deplete inorganic carbon in the surface layer (Ibelings and Maberly, 1998). It has been suggested that cyanobacteria are very efficient in carbon fixation and have a competitive advantage over eukaryotic algae once low pCO_2 is established (Shapiro 1973; Shapiro 1997). Though, this competitive advantage hypothesis is not always supported (e.g. Verschoor *et al*. 2013). Furthermore, buoyant cyanobacteria may intercept CO_2 from the atmosphere and use it for their own growth before it can diffuse in to the subsurface where nonbuoyant phytoplankton live (Pearl and Huisman 2009). However, light interception at the surface may be the most important factor in suppressing growth of eukaryotic algae once a surface bloom is established (Shapiro 1973; Klemer 1989).

Depletion of inorganic carbon at the surface layer occurs when the demand for CO_2 in a bloom exceeds the supply of CO_2 from the atmosphere. Consequently, the rate of inorganic carbon supply effectively controls the rates of net photosynthesis of the bloom (Ibelings and Maberly, 1998). Even the diffusive influx resulting from air that is a tenfold of present day pCO_2 generally cannot satisfy the demand for DIC of an intensive bloom (Ibelings and Maberly, 1998). Freshwater environments are generally poorly buffered and experience large shifts in pH, especially during bloom formation. This is accompanied by a shift in the ratio of carbon species. The restricted diffusion of atmospheric CO_2, variability of DIC and varying proportions of the carbon species impose a challenge for the carbon acquisition of bloom forming cyanobacteria (Badger *et al*. 2006). Which impacts increased pCO_2 has on cell physiology and growth rates of different cyanobacteria genera, and how this may alter the phytoplankton community is subject of many recent studies. Understanding how mechanisms for carbon acquisition respond to environmental conditions is a perquisite for understanding the effects of climate change on bloom forming cyanobacteria.

Carbon Acquisition

Carbon fixation by phytoplankton takes place by the enzymatic CO_2 fixation, catalyzed by Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). RuBisCO catalyzes both the CO_2 fixating reaction, carboxylation, as well as the oxygenation, a wasteful side-reaction (e.g. Heldt and Piechulla, 2011). A parameter describing the ability of RuBisCO to discriminate between CO_2 and O2 is the specificity factor (Badger *et al.* 1998; Tortell 2000; Bowsher *et al.*, 2008). It is defined as $V_{CO2}.K_{O2}/V_{O2}.K_{CO2}$, in which V is the maximum reaction rate and K is the half saturation constant, also called the substrate affinity. Larger values, as observed in cyanobacteria, indicate a greater selectivity for CO_2 and therefore a higher capacity for carboxylation relative to oxygenase. The use of RuBisCO as catalyst for biomass production by cyanobacteria probably dates back to 2.4-3.5 billion years ago (Whitney *et al*. 2011, Rae *et al*. 2013). It has been suggested that the rise of oxygen in the early

atmosphere led to considerable evolutionary pressure to increase the carboxylation efficiency of the enzyme (Jorden and Ogren, 1981; Tortell, 2000). Despite high values of RuBisCO specificity found in some phytoplankton taxa, direct use of concentrations of CO_2 and O_2 resembling the present-day atmospheric concentrations does not result in efficient photosynthesis. Although RuBisCO is an inefficient enzyme for CO_2 fixation with the present day atmosphere, it is still the most abundant protein in the world (Ellis, 1979, Rae *et al*. 2013, Carmo-Silva *et al*. 2015). To compensate for the low RuBisCO efficiency, cyanobacteria have developed CO_2 concentrating mechanisms (CCMs) which can elevate the CO_2 conditions around RuBisCO by up to a thousand-fold (Badger, 2003). These mechanisms allow RuBisCO to fix CO_2 efficiently, even at low environmental pCO_2 and high O_2 by promoting the carboxylase reaction and suppressing the oxygenase reaction (Rae *et al*. 2013). In fact, most phytoplankton species possess a CCM, but there is a large variation between phytoplankton species in the effectiveness of their CCM (Rost *et al*. 2003, Pessarakli, 2016).

The most important traits of RuBisCO for the process of carbon fixation are the carboxylation turnover rate and the affinity for inorganic carbon (Shih *et al*. 2016). These traits are not independent of each other: there appears to be a trade-off (Shih *et al*. 2016). For example, RuBisCO in cyanobacteria has, compared to other algae, a particularly low affinity for CO_2 and O_2 while it has high turnover rates (Price *et al*. 2008). Since the CCM effectively increases cellular affinity to CO_2 and concentrates CO_2 in the vicinity of RuBisCO, the enzyme can work near optimal efficiency. While CO_2 is the required form of carbon that can serve as substrate for RuBisCO, many phytoplankton species are capable of simultaneous uptake of CO_2 and HCO_3^- (e.g. Rost *et al*. 2003, Kranz *et al*. 2010). While CO_2 can be taken up by cells by passive diffusion, HCO_3^- requires active uptake. This can occur by direct uptake or by the use of extracellular carbonic anhydrase that enhances the conversion of HCO_3^- to CO_2, facilitating diffusive CO_2 uptake. An advantage of using HCO_3^- as carbon source is that it is often abundantly available in aquatic environments. However, an important downside for active uptake is that it requires considerable amounts of energy. Therefore often CO_2 is the preferred substrate (Rost *et al*. 2003). Moreover, among phytoplankton there is a great variety in the capability to use HCO_3^- (Hein, 1997). While some species predominantly take up CO_2, other species, like the cyanobacterium *Trichodesmium erythraeum*, rely heavily on HCO_3^- uptake (Kranz *et al*. 2009, Kranz *et al*. 2010). In fact, most cyanobacteria have active uptake systems for HCO_3^- (Price *et al*. 2008), though they may not all rely as heavily on HCO_3^- as *Trichodesmium*. Cyanobacteria often take up both CO_2 and HCO_3^- efficiently since, in addition to active HCO_3^- uptake systems, cyanobacteria also have active uptake systems for CO_2.

Cyanobacteria possess in general 5 different active carbon uptake systems: Two for CO_2 uptake and three for HCO_3^- uptake (Price *et al*. 2008). These systems have different affinities and flux rates and have a different effectiveness under a variety of carbon conditions (Sandrini *et al*. 2014). However, some cyanobacteria are specialized and possess only three or four out of the five different carbon uptake systems, which allows them to outcompete generalists under specific conditions (Price *et al*. 2008, Sandrini *et al*. 2014). This specialization may also occur within a species, as is shown for different strains of *Microcystis aeruginosa* (Sandrini *et al*. 2014).

Carbon isotopes

The carbon isotopic composition of biomass can provide information concerning the processes involved in CO_2 fixation. For example, C_3 plants can be well distinguished from C_4 plants by their $\delta^{13}C$, representing their different photosynthetic pathway (Ehleringer, 1991). Changes in the carbon acquisition of phytoplankton may be reflected in their isotopic composition. For instance, Laws (1998) used carbon isotopes to conclude that *Emiliania huxleyi* primarily used HCO_3^- as carbon source at low growth rates while it mainly used CO_2 at high growth rates.

The carbon isotope composition ($\delta^{13}C_{sample}$) is noted by the delta notation, relative to the Vienna-PeeDee belemnite standard:

$$\delta^{13}C_{\text{sample}} = \frac{(^{13}C/^{12}C)_{\text{sample}}}{(^{13}C/^{12}C)_{\text{PDB}}} \qquad (1)$$

Where $(^{13}C/^{12}C)_{sample}$ refers to the measured isotopic ratio of the sample and $(^{13}C/^{12}C)_{PDB}$ is the isotopic ratio of the Vienna-PeeDee belemnite standard. Carbon isotope discrimination, or fractionation, has been shown to be strongly affected by pCO_2. This dependence of fractionation to pCO_2 is of particular interest in the field of paleoclimatology, in which reconstruction of ancient pCO_2 is of major interest. Fundamental knowledge about how cellular processes fractionate and how this depends on other environmental conditions is required to make accurate reconstructions.

The main process causing fractionation in photosynthetic organisms is the enzymatic CO_2 fixation catalyzed by RuBisCO. This process strongly discriminates against ^{13}C, which in most cases leads to a large negative $\delta^{13}C$ of biomass. The $\delta^{13}C$ of biomass is however, next to cellular processes, also determined by the $\delta^{13}C$ of the carbon source, which can vary considerably per location and in time. If the isotopic composition of the carbon source is known, isotopic fractionation is determined, which is expressed relative to the carbon source:

$$\varepsilon_{\text{p}} = \frac{\delta^{13}C_{CO_2} - \delta^{13}C_{POC}}{1 + \delta^{13}C_{POC} \cdot 10^{-3}} \qquad (2)$$

In which ε_p is the fractionation (per mile, ‰), $\delta^{13}C_{CO2}$ is the isotopic composition of the carbon source and $\delta^{13}C_{POC}$ is the isotopic composition of the biomass (Freeman and Hayes 1992). Equation 2 shows that for the calculation of fractionation, the isotopic composition of the carbon source is required. As was indicated before, phytoplankton has been shown to be able to use both CO_2 and HCO_3^- as carbon source. These compounds strongly differ in isotopic composition due to isotopic fractionation at thermodynamic equilibrium. In this so-called equilibrium fractionation, the heavier isotope preferentially remains in the most strongly bound compound. In the case of the carbonate system, this means HCO_3^- is more ^{13}C enriched than CO_2 in isotopic equilibrium. Differences or shifts in the proportion of CO_2 to total carbon uptake (CO_2+HCO_3^-) may therefore affect the isotopic composition of phytoplankton.

In any unidirectional process, such as carboxylation by RuBisCO, fractionation is only expressed if the reaction is incomplete. With a complete reaction, all of the reactant is transformed into the product. This way the isotopic composition of the product is equal to the composition of the reactant. Alternatively, if there is fast refreshment of the reactant, the product can be strongly depleted in the heavy isotope. For carboxylation by RuBisCO this means that its strong intrinsic fractionation may not

be expressed if all the carbon that enters the cell is used for carbon fixation. If instead the pool of carbon is refreshed rapidly by new carbon entering the cell and old carbon leaving the cell (leakage), the fixed carbon is strongly depleted in ^{13}C. This process is described in the classical model by Sharkey and Berry (1985). This model was later extended by Burkhardt *et al*. 1999a, to account for two possible carbon sources, by adding the term R_{HCO3^-}, resulting in the following expression for ε_p :

$$\varepsilon_p = R_{HCO_3^-} . \varepsilon_s + \varepsilon_{rub} . L \qquad (3)$$

In this model, R_{HCO3^-} is the fraction of bicarbonate to total carbon taken up by the cell, ε_s represents the equilibrium discrimination between CO_2 and HCO_3^-, ε_{rub} represents the kinetic fractionation by RuBisCO and *L* represents the leakage out of the cell, which is the ratio of efflux over influx:

$$L = \frac{F_{out}}{F_{in}} \qquad (4)$$

The equilibrium discrimination between CO_2 and HCO_3^- (ε_s) has been described by Mook *et al*. 1974, which is a function of temperature and has a value of ~9% in common experimental settings. Retrieving an appropriate value for ε_{rub} is difficult, as data on RuBisCO kinetics are sparse and limited to few species. Additionally, there are many different types of RuBisCO, which may differ in their kinetic fractionation. Plants and the majority of the cyanobacteria, including *Microcystis aeruginosa*, possess a type 1B RuBisCO (Tabita *et al*. 1999; Shih *et al*. 2016). The intrinsic fractionation of type 1B RuBisCO has been investigated in *Spinacea oleracea* (spinach) and *Anacystis nidulans* (a freshwater cyanobacterium). However even within this RuBisCO type there is a large difference in fractionation: the carbon fractionation of *Spinacea* and *Anacystis* are 29‰ and 22‰, respectively (Roeske and O'Leary, 1984; McNevin *et al*. 2006).

Besides carboxylation of RuBisCO, fractionation may also occur with fluxes of CO_2 and HCO_3^- over the cell membrane. The fractionation associated with these fluxes is however generally considered to be very small (<1‰) (Goericke *et al*., 1994; Keller and Morel, 1999; Burkhardt *et al*. 1999a). Additionally, fractionation of CO_2 influx and efflux have an opposite net effect, resulting in a net very small contribution to ε_p values, compared to the large fractionation associated with RuBisCO carboxylation. Fractionation occurring during carbon flux over the cell membrane is therefore considered negligible in this study.

There are strong differences in fractionation between species. Differences in CO_2 permeability of the cell membrane, enzymatic fractionation, cellular carbon content and cell surface to volume ratio have been suggested to be potentially important factors causing variation between species (Burkhardt *et al*. 1999b). How these factors affect fractionation is currently poorly understood. A better understood factor that affects fractionation is the growth rate. In fact, it has been attempted to estimate growth rates, or reconstruct them, from fractionation values (Francois *et al*. 1993, Laws 1998, Burkhardt *et al*. 1999a,b). In some cases growth rate has a larger effect on fractionation than pCO_2 (e.g. Burkhardt *et al*. 1999b, Rost *et al*. 2002). Therefore, often the ratio of μ/CO_2 is used to interpret isotope data, describing the CO_2 demand over supply and to account for their combined effects (Laws *et al*. 1997; Rost *et al*. 2002). On the basis of a simple diffusion model Burkhardt *et al*. (1999a) suggested that deviations from an inverse linear relationship between ε_p and μ/CO_2 indicate active uptake of carbon and thus the operation of CCMs.

Effects of pCO_2

While phytoplankton may benefit from increased pCO_2, there may be negative consequences to the concurrent decreased pH. The positive or negative consequences strongly varies between species (Schippers *et al*. 2004). Species with a low affinity for HCO_3^- do not seem carbon saturated at 350 ppm CO_2 and may therefore benefit strongly from increased pCO_2 (Schippers *et al*. 2004). However, calcifying species with a high sensitivity for pH may be negatively affected (Riebesell *et al*. 2000a). These different responses between species show that increased pCO_2 may induce major shifts in the phytoplankton community (Schippers *et al*. 2004; Tortell *et al.* 2008; Trimborn *et al.* 2013). Competition experiments have shown that different strains may respond differentially to increased pCO_2, which may shift the genotype composition within populations (Van de Waal *et al*. 2011).

For cyanobacteria, increasing levels of pCO_2 may be beneficial. Laboratory studies have shown that elevated atmospheric pCO_2 promotes cyanobacteria growth and biomass build-up (Verschoor *et al*., 2013; Verspagen *et al*., 2014b). This effect mainly occurs when nutrients are replete and when high cyanobacteria population densities drive the system to carbon limitation. The increase in pCO_2 may then alleviate the carbon limitation and shift the system to light limitation, promoting biomass build-up (Van de Waal *et al.* 2009; Verspagen *et al*., 2014b). Systems that are limited by nutrients rather than carbon, would not necessarily benefit from increased pCO_2. Increased pCO_2 could shift the resource limitation from carbon limitation to nutrient limitation (Van de Waal *et al.* 2009; Verschoor *et al*., 2013; Verspagen *et al*., 2014a). This shift does not cause a significant increase in biomass, but it can shift the elemental composition to higher carbon:nutrient ratios (Verschoor *et al*. 2013, Verspagen *et al*. 2014a). This may have considerable impacts on the food quality for grazing zooplankton (Sterner and Elser 2002, Van de Waal *et al*. 2010a).

Like all autotrophs, phytoplankton take up carbon and nutrients separately (Sterner and Elser, 2002). Consequently, phytoplankton have a flexible stoichiometry. Their elemental composition can be greatly affected by the resource availability in the environment. Large shifts in stoichiometry can occur when a resource is in great excess relative to another, resulting in C:N, C:P, N:P ratios deviating strongly from the Redfield ratio (C:N:P=106:16:1). Changes in C:N:P occur due to changes in the abundance of various organic macromolecules or from accumulation of nutrient/energy reserves (Geider and Roche 2002). For example, nitrogen limitation can cause an increase in the C:N ratio. This occurs mainly by an increase in carbohydrate reserves, but also by a decrease of nitrogen-rich compounds as RuBisCO (Turpin 1991). A number of studies has shown that an increase in pCO_2 may greatly enhance C:N ratios (Riebesell *et al*. 2007; Verschoor *et al*. 2013; Verspagen *et al*. 2014a). This occurs in particular when carbon remains replete and nitrogen is limiting (Verspagen *et al*. 2014a).

A change in pCO_2 may also have considerable consequences on the carbon acquisition of phytoplankton employing a CCM. The CCM has been shown to respond to available inorganic carbon concentrations. For example, experiments performed with *Chlorella ellipsoidea* by Matsuda and Colman (1995) have shown that a critical level of dissolved CO_2 concentration can induce the operation of CCMs. High concentrations of CO_2 in the medium can lead to a repression of active transport, while low concentrations can induce it (Matsuda and Colman 1995, Matsuda *et al*. 1998). This change in CCM activity is associated with a change in affinity for inorganic carbon. Cyanobacteria are shown to increase their affinity for inorganic carbon at low concentrations of inorganic carbon (McGinn *et al*. 2003, Ma and Gao 2014). This change in affinity for inorganic carbon is mainly due to

the induction of the various CO_2 and HCO_3^- transporters at low carbon availability (McGinn *et al*. 2003, Badger *et al*. 2006). At low pCO_2 this allows cyanobacteria to deplete CO_2 further to very low levels. Additionally, with increased CCM activity at low pCO_2 / high pH, the lack of available CO_2 can be compensated by an increase in the proportion of HCO_3^- taken up (Rost *et al*. 2003; Kranz *et al*. 2010).

Research subject and hypothesis

To summarize, an increase in pCO_2 in lakes, driven by the anthropogenic increase of atmospheric CO_2 or increase in allochtonous DOC respiration may have large consequences on the growth and cellular processes of harmful cyanobacteria. In cyanobacteria blooms, during which inorganic carbon is depleted and nitrogen is replete, an increase in pCO_2 could alleviate carbon limitation, leading to an increase in biomass build-up. It may also induce nitrogen limitation and cause an increase in carbon:nitrogen ratios. Such an increase in carbon:nutrient ratios is indicative of major cellular changes such as accumulation or reduction of nutrient/energy reserves and changes in the abundance of compounds like RuBisCO. Carbon acquisition may be influenced by elevated pCO_2 as it may cause a down-regulation of CCMs, but also by changes in relative nitrogen availability, as this is required for RuBisCO. All-in-all, changes in pCO_2 may significantly alter cyanobacteria growth and cellular processes, but the nature of these changes is strongly dependent on the prevailing nutrient conditions. Isotopic fractionation may provide information concerning the changes in carbon acquisition. Furthermore, from a different perspective, fundamental knowledge on CO_2-driven and nutrient-driven changes on cellular processes affecting isotopic fractionation is required for the validation and refinement of carbon isotope values as a CO_2 proxy. In this research we therefore investigate changes in growth, carbon uptake, and elemental composition driven by the availability of inorganic carbon and nitrogen.

We investigated the response of three strains of the toxic, bloom forming freshwater cyanobacterium *Microcystis aeruginosa* to a wide range of pCO_2 that can occur in natural environments, in nitrogen replete and depleted conditions. We expected an increase in growth rate and biomass build-up with increasing levels of pCO_2 in nutrient replete conditions, especially from the point where pCO_2 may be limiting. In contrast, in nitrogen deplete conditions we expected no large change in biomass build-up, but do expect an increase in carbon:nutrient ratios. We furthermore predicted a general increase in carbon isotope fractionation with increasing pCO_2, reflecting changes in carbon acquisition and the activity of the CCM.

Material and Methods

Three different *Microcystis aeruginosa* strains, PCC 7820, NIES 1099 and HUB 524 were incubated at five different pCO_2 levels, which at the end of the experiment resulted in measured pCO_2 of 6.2-101.2 µatm; 112-267 µatm; 509-825 µatm; 1326-1697 µatm; 2126-2737 µatm. They were grown as single cells in monoculture, but were not axenic. Continuous input of moist air at the bottom of the culture vessels caused homogeneous mixing and turbulence, which prevented settling of the cells. Microscopic inspection confirmed that cells were grown generally as single cells. The gas was a mixture of N_2, O_2 and CO_2 for the lower than ambient CO_2 treatments. Higher CO_2 treatments consisted of pressurized air and specific amounts of additional CO_2 gas. The gasses were combined using a Brooks® Smart II mass flow controller, which controlled the gas pressure of the components and the mixture. The pCO_2 levels of the treatments were confirmed by calculation of the pCO_2 in the excel macro CO2sys (Pierrot *et al*., 2006), using the alkalinity and pH of uninoculated medium after equilibrating with a continuous flow of the gas mixture. DIC uptake of the cyanobacteria resulted in offsets from these treatments and the final pCO_2 was calculated with alkalinity and pH measured at the end of each experiment. The organisms were grown under saturating light conditions, with a light intensity of 100 ±15 µmol photons $m^{-2}s^{-1}$ supplied by daylight tubes (PL-L 24W/840/4p, Philips) at a light:dark cycle of 16:8 h. The temperature was maintained at 22±1°C. Dilute batch experiments as well as chemostat experiments were performed with the same gas mixture, leading to similar, but not equal pCO_2 conditions. This is due to differences in population densities. In the dilute batch experiments, nutrients were replete so the cyanobacteria could grow at their maximum growth rate. In this exponential growth phase the cell density increases rapidly while the nutrient concentration declines. In the chemostat experiments, nutrients were deplete and growth was limited by resource availability. In this system a steady state is reached, where the cell density is stabilized and the limiting nutrient is depleted to its minimum concentration. This minimum concentration is determined by the nutrient affinity, maximum uptake rate and cellular quota of the species (Tilman, 1982; Litchman *et al.* 2007). At steady state growth is equal to the dilution rate. Therefore, the responses to pCO_2 in the different chemostat experiments were independent of growth rate. Furthermore, in steady state nitrate consumption equals the supply rate. This allows a continuous culture to reach and maintain N-limited conditions. The set-up of both types of experiments are explained below.

Batch experiments

The three *Microcystis* strains were grown in 1 liter bottles, three replicates per strain. The cyanobacteria were grown in modified WC medium (Kilham *et al*. 1998), adjusted to 500 µmol L^{-1} HCO_3^-. Before starting the experiments the organisms were acclimated to the experimental conditions for 7 generations, after which the acclimated culture was added to fresh medium. On a daily basis pH, cell count, biovolume and chlorophyll a content were measured. After typically 2-3 generations (4 days) the cells were harvested around the middle of the light period and additional samples were taken for the determination of alkalinity, nutrients, dissolved inorganic carbon (DIC) and particulate organic C, N and P, including $\delta^{13}C$ of DIC and organic C.

Chemostats experiments

The cyanobacteria were grown in chemostats with a working volume of 1.7 liter and an optical path length of 5 cm (Huisman *et al*. 2002). They were grown in modified, nitrogen limited, WC medium (Kilham *et al*. 1998), adjusted to 500 µmol L^{-1} HCO_3^-. and 200 µmol L^{-1} NO_3^-. Nutrients were added continuously with a dilution rate of 0.2 d^{-1}. Temperature was maintained by a cooling finger. Cultures were acclimatized for a period of 2-3 weeks. Treatments were run until steady state was reached, defined as the level at which the biomass stabilizes and remains within the bounds of 10% variation for over a week. The pH, cell count, biovolume and chlorophyll a was measured three times a week. Alkalinity was measured on average once a week. At steady state, samples were taken for residual nutrients. On the final day, around the middle of the light cycle, additional samples were taken for measurements of DIC and particulate organic C, N and P. After a treatment was finished, >80% of the volume was removed, vessels were refilled with medium, and cultures were acclimated again for a period of 2-3 weeks after the next pCO_2 level was set.

Measurements

After samples were taken, the pH and temperature were measured with a WTW pH/conductivity 340i, while the sample was being stirred by a magnetic stirrer. Cell counts and biovolume were measured with a Beckman Coulter Multisizer™ 3 counter. Samples for alkalinity were filtered over a pre-washed Whatman® GF/F glass microfiber filter (~0.7 um) and then analyzed on a Titralab® titration manager TIM840. Samples for nutrients were filtered over a 0.4 µm Satorius membrane filter, stored at -20^{o}C and analyzed on a QuAAtro39, Seal Quaatro, Analytical Ltd. autoanalyzer. DIC samples were taken at the same way as these nutrient samples. The DIC samples were then analyzed for their isotopic content according to Salata *et al*. 2000 using a GC-IRMS at Utrecht University with an accuracy of ~0.1‰. Particulate organic carbon, nitrogen and phosphorus was determined by filtering 10-60mL of the culture suspension to get approximately $1*10^8$ μm^3 biovolume on the filter, followed by drying of the filters at 60^{o}C and then analyzed for carbon and nitrogen on a Thermo interscience NC analyser FlashEA®1112. Particulate organic phosphorus samples were ashed in an oven at 500°C, digested with 2.5% persulfate in an autoclave at 121°C and finally analyzed on a QuAAtro39, Seal Quaatro, Analytical Ltd. autoanalyser.

Calculations

The 24-hour specific growth rates (µ) for the dilute batch experiments were determined based on cell densities by means of a best exponential fit through all measurements, according to:

$$N_t = N_0 \,.\, e^{\mu t}$$

Where N_t represents the cell density at time t, N_0 represents the cell density at the first measurement and µ refers to the growth rate. Carbon and nitrogen production rate was calculated based on the cellular carbon and nitrogen quota (Q), multiplied with µ. Carbon production rate is sometimes also referred to as carbon-specific growth rate (μ_c).

For parameters of the batch experiment (n=3) that were calculated, such as μ_c and C:N ratios, error propagation was applied using the standard deviations of the measured parameters, SD_{var1} and SD_{var2} and their respective averages avg_{var1} and avg_{var2} while assuming independence of variance, according to:

$$SD_{propegated} = \sqrt{(SD_{var1} - avg_{var1})^2 + (SD_{var2} - avg_{var2})^2} * value$$

Propagated standard deviations are shown in the figures.

To determine fractionation (equation 3) the $\delta^{13}C$ of CO_2 is required. This was calculated from $\delta^{13}C$ of DIC using mass balance equations as described in Zeebe and Wolf-Gladrow, 2001. Required values for equilibrium fractionations between the components in the carbonate system were retrieved from Mook *et al*. 1974 and Zhang *et al*. 1995. They developed equations that describe this equilibrium fractionation as a function of temperature. The equilibrium value for CO_2 and bicarbonate is -9.32‰ at our experimental temperature (22^{o}C) (Mook *et al*. 1974) and the equilibrium value for CO_2 and carbonate is 6.08‰ (Zhang *et al*. 1995). The concentrations of CO_2, HCO_3^- and CO_3^{2-} were determined in CO2sys using the alkalinity and pH measured at the end of each experiment.

Results

Carbon chemistry in the experimental cultures was not only affected by the applied CO_2 treatment, but also by cyanobacteria growth. Compared to the batch cultures, populations in the chemostats reached higher population densities. This resulted, despite similar gas supply, in a larger drawdown of CO_2 in the chemostats. Especially with the lowest CO_2 treatment (target: 50 ppm), the pCO_2 of the medium was 3-11 times lower in the chemostat cultures than in the batch cultures. At the highest pCO_2 treatment (target: 1600 ppm) one of the chemostat populations with the PCC-7820 strain washed out, consequently for this strain only data for a single chemostat is available at the highest pCO_2 treatment.

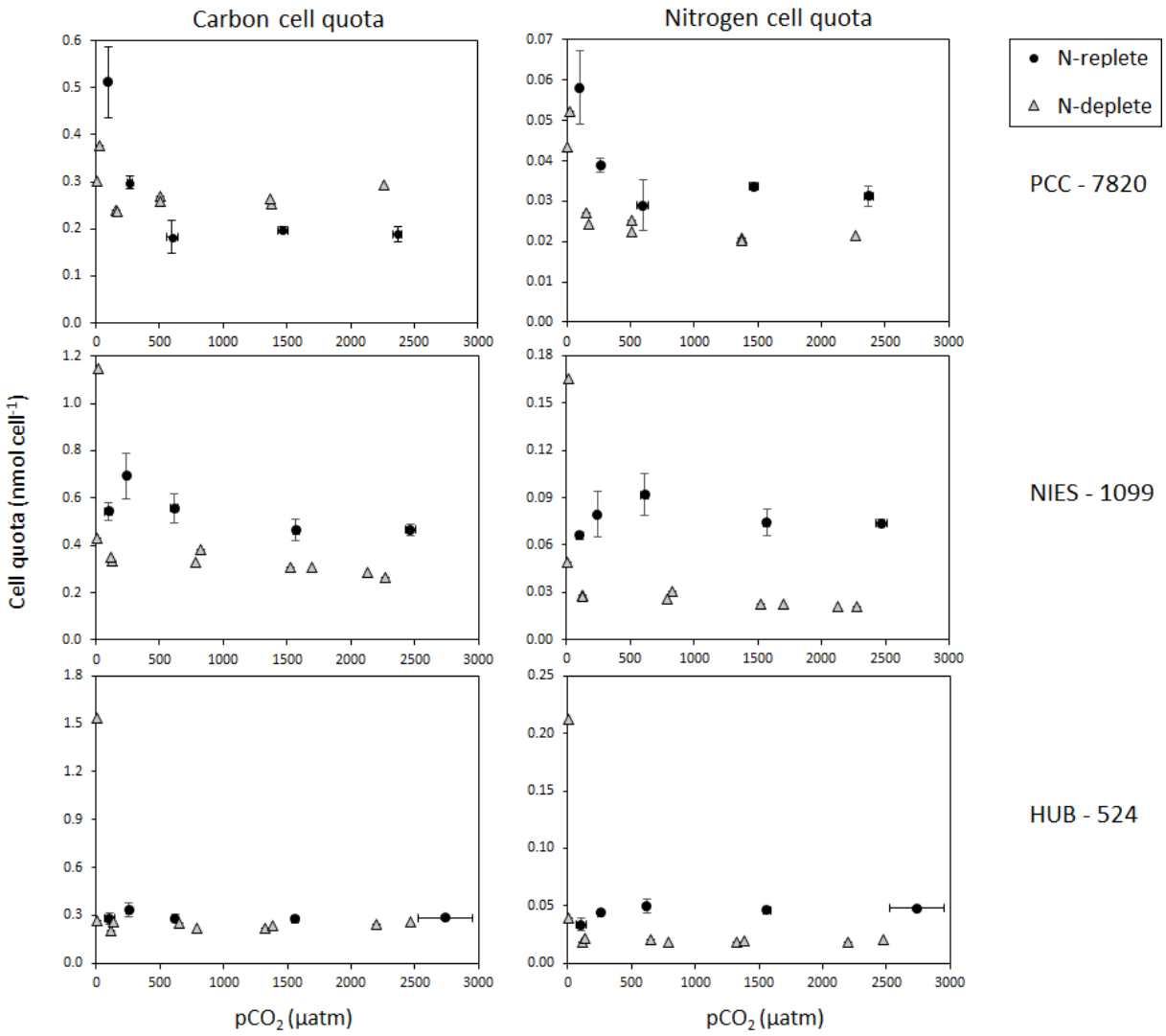


Figure 2. Carbon and nitrogen cell quota. *For nitrogen replete treatment, 95% confidence intervals are shown (n=3). For nitrogen deplete treatment, all individual data points are shown (n=1).*

Changes in both carbon and nitrogen cell quotas were observed with increasing pCO_2, especially in the lower CO_2 range (fig.2). Nitrogen conditions seem to have a major effect on both carbon and nitrogen cell quotas as well, and seem to interact with the effects of elevated pCO_2. Specifically, in nitrogen replete conditions (+N), carbon cell quotas decreased with increasing pCO_2 for the PCC-7820 and the NIES-1099 strains. While in nitrogen deplete conditions (-N), apart from increased carbon cell quota at the lowest pCO_2 treatment, no large and no consistent trend is observed in the different strains. Nitrogen limitation systematically resulted in lower nitrogen cell quota. Accounting for cell size by taking quota per biovolume instead, results in similar trends with increasing pCO_2 (supplementary fig. 1). Carbon quota of showed contrasting responses to N limitation between the strains, particularly at higher pCO_2, with an increase in PCC-7820, decrease in NIES-1099 and no change in HUB-524.

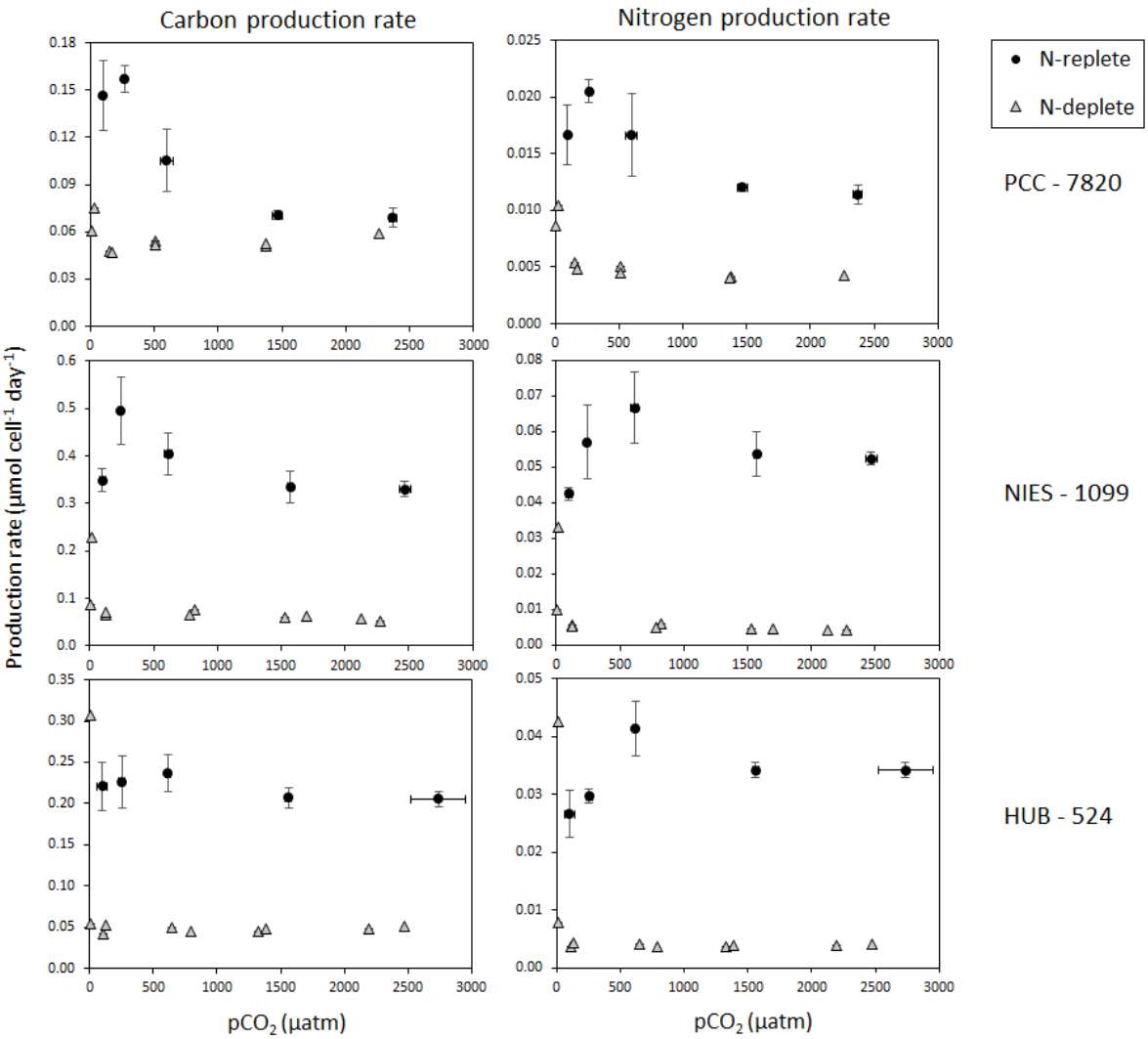


Figure 3. Carbon and nitrogen production rates. *For nitrogen replete treatment, 95% confidence intervals are shown (n=3). For nitrogen deplete treatment, all individual data points are shown (n=1).*

Carbon and nitrogen production rates (fig.3) and growth rates (supplementary fig. 2) in +N show an increase from the lowest pCO_2 treatment to an optimum between 240-616 µatm. Growth rates in +N decreased for all strains for pCO_2 >1465 µatm (supplementary fig. 2). This is also reflected in carbon and nitrogen production rates, which decreased at high pCO_2 (fig. 3). The population densities in -N increased with increasing pCO_2 (supplementary fig. 3). Carbon and nitrogen production rates in -N show an initial decrease at the lowest pCO_2, but towards higher pCO_2 no general trend is visible in the different strains. Nitrogen limitation results in much lower overall production rates and a low sensitivity of these rates to pCO_2.

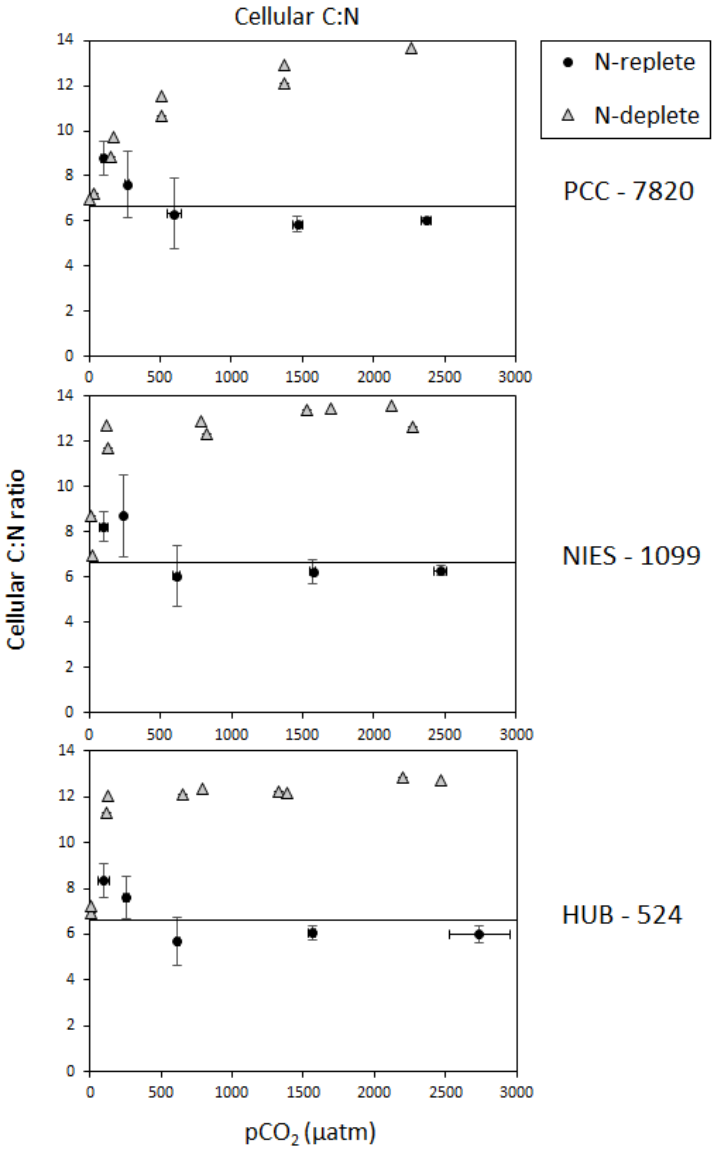


Figure 4. C:N ratios. *For nitrogen replete treatment, 95% confidence intervals are shown (n=3). For nitrogen deplete treatment, all individual data points are shown (n=1). The line represents Redfield ratio (C:N= 6.625)*

For all strains there is a general trend of decreasing C:N with increasing pCO_2 under excess nitrogen conditions, which levels off to just below the Redfield ratio at intermediate pCO_2. An opposite trend is observed under nitrogen deplete conditions. While at low pCO_2 C:N values approach Redfield ratio, at high pCO_2 nitrogen deplete conditions resulted in up to two times higher cellular C:N ratios in nitrogen deplete than in nitrogen excess conditions. At very low and very high pCO_2 levels, the cellular C:N seems similar in all strains. However, under nitrogen deplete conditions the strain PCC-7820 responds more gradual to increasing pCO_2.

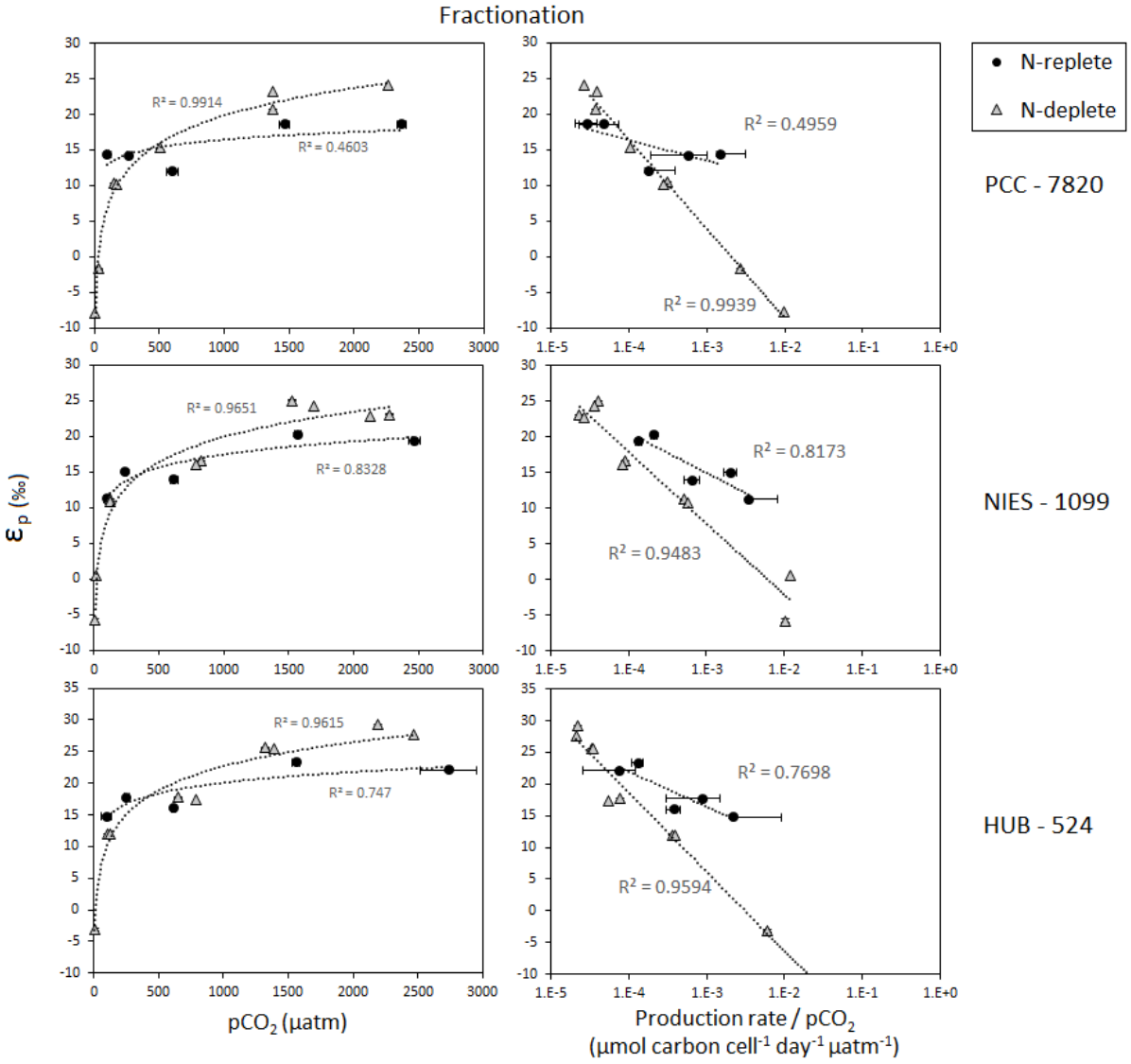


Figure 5. Carbon isotope fractionation. *For nitrogen replete treatment, 95% confidence intervals are shown (n=3). For nitrogen deplete treatment, all individual data points are shown (n=1). Trend lines are logarithmic best fits. The regression results are provided in supplementary tables 1+2.*

Fractionation increases logarithmically with increasing pCO_2. With high pCO_2 the fractionation is high and the ε_p approaches the value of kinetic fractionation by RuBisCO in -N (Spinach RuBisCO: 29‰, Roeske and O'Leary, 1984). While with low pCO_2 the ε_p is negative under N limited conditions and it approaches the value of equilibrium discrimination of bicarbonate and dissolved CO_2 (-9.3‰, calculated with T=22 °C from Mook *et al*. 1974). In nitrogen deplete conditions the range of ε_p is much higher than in nitrogen excess conditions. The sensitivity of carbon fractionation to pCO_2 is much higher in -N than in +N. The relation between fractionation and carbon demand/carbon supply is given by μ_c/pCO_2 (figure 5, right), which is well represented by a logarithmically decreasing line in both nitrogen treatments. This relation also shows a steeper slope in –N than in +N.

Discussion

Response of cell quota and stoichiometry

This research investigated the response of *Microcystis aeruginosa* to different pCO_2, ranging from 6-2737 µatm, in excess nitrogen as well as nitrogen deplete conditions. Changes in pCO_2 strongly affected the elemental composition of *Microcystis*. Cell quotas were affected by the imposed pCO_2 conditions and by the availability of nitrogen. Carbon and nitrogen cell quota showed a comparable direction in response, with an initial sharp decrease with pCO_2 that levelled off above close to present day pCO2. However, under nitrogen limited conditions, the decreases in N were stronger than of C, while under N replete conditions, the decrease in C was stronger than in N. This resulted in an opposite response of C:N ratios to increasing pCO_2 (fig.4). Such changes are often observed in photoautotrophs, and reflect their flexible stoichiometry. This flexibility results from the capability of phytoplankton to take up carbon and nutrients separately (Sterner and Elser, 2002). However, there are limits to the flexibility as the metabolism of carbon and nitrogen are tightly coupled (De Marsac *et al*. 2001). For example, the synthesis of amino acids involves both the carbon and nitrogen metabolism. A pulse of nitrogen may promote the production of arginine and generate a high demand for 2-oxoglutarate, which is a product of the carbon metabolism required for arginine synthesis (e.g. Van de Waal *et al.* 2010b). This shows interactions between nitrogen assimilation and carbon metabolism can occur during compound synthesis (De Marsac *et al*. 2001). This coupling of carbon and nitrogen metabolism may explain the similarity between the response of carbon and nitrogen cell quotas to increasing pCO_2, affecting the general shape of the graphs. For instance, it has been shown that under carbon limitation the rates of nitrogen uptake are decreased (De Marsac *et al*. 2001). This may also be reflected by our data, as nitrogen production rates strongly decreased under to low pCO_2 under excess nitrogen conditions (fig. 3). Nitrogen cell quota for NIES-1099 and HUB-524 also decreased under these conditions. Interestingly, under nitrogen deplete conditions, the opposite is observed, with increased nitrogen cell quota and increased nitrogen production rates at low pCO_2 of 6-29 µatm. This occurs together with a large decrease in population density at these low pCO_2. The strong drawdown of pCO_2 and a concurrent decrease in population density indicates carbon limitation. Furthermore at low pCO_2, C:N ratios approach Redfield ratio (fig. 4).

It is clear that major changes occur by the combined effect of low pCO_2 and nitrogen deplete conditions. Under such conditions, carbon, nitrogen or both may be limiting. The large changes in stoichiometry with increasing pCO_2 indicate a strong sensitivity to pCO_2, especially in the nitrogen depleted treatment at very low pCO_2. Here, NO_3^- levels in the medium are shown to increase, which may suggest nitrogen is not limiting in this situation. Alternatively, lower pCO_2 levels reduce the efficiency by which nitrogen can be acquired, and thereby resulting in an increased residual NO_3^-.

While at the lowest pCO_2 treatment, CO_2 may be limiting in the nitrogen depleted treatment, the chemostats switch to nitrogen limitation at the higher pCO_2 treatments. This switch from carbon limitation to nitrogen limitation has been described and reported by Verspagen *et al*. (2014a). They describe shifts in the limiting resource by a model, supported by chemostat experiments. They show that elevated pCO_2 can cause a shift from carbon to nitrogen limitation, thereby causing a decrease in dissolved nitrogen concentrations, and an increase in C:N ratios as well as population densities.

Our results are largely in line with their findings. The opposite trend in C:N under nitrogen replete conditions, however, is not represented by the model by Verspagen *et al*. 2014a. After a close inspection of their experimental chemostat data, however, there might be an optimum in C:N around 100 ppm pCO_2, which compares with our lowest pCO_2 treatment and its associated maximum C:N.

Response of growth and carbon acquisition

As the results in the previous paragraph indicate, the responses towards changes in pCO_2 are complex and not linear. Substantial changes in growth and cell quotas with increasing pCO_2 are observed. These changes may be driven by changes in carbon acquisition, since phytoplankton have a strong capability to actively respond to the carbon availability (Matsuda *et al*. 1998, Moroney 1999). While the operation of CCMs is induced at a critical level of dissolved CO_2 concentration for the green algae *Chlorella ellipsoidea*, the induction of CCMs of cyanobacteria seems to be dependent on concentrations of bicarbonate or total inorganic carbon instead (Mayo *et al*. 1986, Matsuda and Colman 1995, Beardall *et al*. 1998). The carbon affinity in *Synechococcus leopoliensis* (cyanobacterium) showed a strong change in the [DIC] range of 10-1000 µM (Mayo *et al*. 1986), which is well in the range of DIC concentrations in our study. Our data indicate that [DIC] generally increased with pCO_2, mainly due to chemical enhancement induced by the rise in alkalinity and pH associated with phytoplankton CO_2 and NO_3^- uptake (Zeebe and Wolf-Gladrow, 2001; Soetaert *et al*., 2007; Wolf-Gladrow *et al*. 2007). DIC concentrations ranged between 500 and 1000 µM. If *Microcystis* has a response to DIC similar to *S. leopoliensis*, repression of the CCM is expected at increasing concentrations of DIC. This can have a major influence on carbon acquisition and the energy balance in the cell (Eichner *et al*. 2015). Under low pCO_2 conditions, active uptake mechanisms for both CO_2 and HCO_3^- can be induced, increasing their substrate affinities (Price *et al*. 2008). Despite CCM induction at low pCO_2, the *Microcystis* strains investigated here, have impaired carbon production rate and growth rate at low pCO_2 (fig. 3, supplementary fig. 2), which is consistent with carbon limitation (Riebesell *et al*. 1993). Additionally, it has been suggested that growth may also be decreased due to a reallocation of energy used for growth to CCM activity (Burnap *et al*. 2015). An increase in CCM activity is primarily due to increased synthesis of various CO_2 and HCO_3^- transporters (Badger *et al*. 2003). These transporters require considerable energy and an upregulation may therefore require additional energy (Burnap *et al*. 2015; energy costs are summarized in Sandrini *et al*. 2015b).

Another possibility for decreased growth at low pCO_2 is photoinhibition. Some species of cyanobacteria are sensitive to light. For instance, the freshwater cyanobacterium *Planktothrix agardhii* has been shown to be inhibited at irradiances of > 100 µmol in non-extreme CO_2 conditions (Tonk *et al*. 2005). In our study, relatively low light is used (100 ±15 µmol photons $m^{-2}s^{-1}$), which is generally below the limit where photoinhibition is observed in cyanobacteria, including *Microcystis* (Coles and Jones, 2000). However, in contrast to Coles and Jones (2000), we apply dilute batch cultures, which maintain a relatively higher light exposure per cell. For growth in very low levels of pCO_2 photoinhibition may occur as excess energy cannot be shunted to CO_2 fixation. This has been shown for the green alga *Chlamydomonas reinhardtii*. When these cells are grown at low pCO_2 they have a fully induced CCM and appear to be more sensitive to photoinhibition than their high-pCO_2 grown counterparts (Falk *et al*. 1992). Similarly, a higher sensitivity for photoinhibition has also been shown to occur in cyanobacteria that experience carbon limitation (Ibelings and Maberly, 1998).

It is striking that growth rates of the investigated strains of *Microcystis* at high pCO_2 are also impaired. At high pCO_2 the CCM can be downregulated to a minimum activity (Price *et al*. 2008) and the cells could then rely more on diffusive uptake, which would decrease the energy costs associated with carbon uptake (Raven and Johnston 1991; Giordano *et al*. 2005; Raven *et al*. 2011). However, the impaired growth rates in our experiments show that this additional available energy apparently cannot be used for growth and that, in fact, growth is hindered somehow in these conditions. A possible explanation for this may be additional light stress. It has been suggested that the CCM may also play an important role in the dissipating of excess light energy, preventing high light stress (Kaplan and Reinhold, 1999; Herrero and Flores 2008). With a downregulated CCM at high pCO_2, the cells might be more sensitive for this.

An alternative explanation for decreased growth rates at high pCO_2 may be found in downregulation of one of the bicarbonate uptake systems. Cyanobacteria are known to use a significant proportion of bicarbonate as their inorganic carbon source. Some species of cyanobacteria e.g. the marine nitrogen-fixer *Trichodesmium* even use HCO_3^- as main carbon source, especially at low pCO_2 (Kranz *et al*. 2010). A switch to HCO_3^- uptake in CO_2 limiting conditions would be strongly beneficial if HCO_3^- is in excess, such as in our experiments. However, *Microcystis* may not be able to rely on HCO_3^- uptake as strongly as *Trichodesmium*. For instance, Verspagen *et al*. (2014b) have suggested that the strain HUB-524 requires uptake of CO_2, as growth is substantially impaired (35% of maximum) under strongly limiting pCO_2 and excess HCO_3^-.

Bicarbonate can be actively taken up by a number of bicarbonate uptake systems: bicA: a low affinity, high flux uptake system; sbtA: a high affinity, low flux uptake system and BCT1: a high affinity, low flux uptake system (Sandrini *et al*. 2015a). The bicA and sbtA are sodium-dependent bicarbonate transporters and BCT1 is ATP dependent (Sandrini *et al*. 2015a). Cyanobacteria possessing all three bicarbonate uptake systems are the most versatile in their response to inorganic carbon (Sandrini *et al*. 2014). In a number of strains, one of the two sodium-dependent bicarbonate transporters is missing. The strains investigated in our study are specialists and possess sbtA as only sodium-dependent transporter (Sandrini *et al*. 2014, unpublished data). Similarly, the strain NIES-843, in the study of Sandrini *et al*. (2014), also possesses sbtA and not bicA. This strain has impaired growth at high pCO_2, just like the strains investigated in our study. Sandrini *et al*. (2014) have suggested that impaired growth may be due to a reduced sbtA expression in high pCO_2. This may also be the case in the strains investigated here. A reduced sbtA expression may lead to decreased reliance of the cell to bicarbonate uptake and an increase in active or diffusive CO_2 uptake.

To summarize, growth rates are negatively affected in low pCO_2 and high pCO_2. This may be due to the effect of pCO_2 on the expression of CCMs (and at low pCO_2, possibly the related energy costs) or this may be due to photoinhibition. In the following parts we investigate changes in carbon acquisition using the carbon isotope data.

Carbon isotope fractionation

The maximum fractionation value found in this study is 29.3‰, in the HUB-524 strain. This is much higher than the intrinsic fractionation of RuBisCO of *Anacystis nidulans* (22‰; McNevin *et al*. 2006). The RuBisCO in *Microcystis* may have a high intrinsic fractionation, perhaps comparable with RuBisCO

from spinach (29‰; Roeske and O'Leary, 1984). Alternatively, carbon fixation by RuBisCO may not be the only process that discriminates against ^{13}C. Yet, fractionation by most other processes in phytoplankton is considered to be small, but will add uncertainty (Goericke *et al*. 1994). There is a general increase of ε_p with increasing pCO_2. This trend of ε_p with increasing pCO_2 is observed in many different kinds of phytoplankton and is generally caused, as equation 3 describes, by a decrease in HCO_3^- relative to total carbon uptake (R_{HCO3}-), and/or an increase in leakage (Sharkey and Berry 1985; Burkhardt *et al*. 1999a; Hoins *et al*. 2016a). Initially there is a strong sensitivity of ε_p to changes in pCO_2, while at high pCO_2 this levels off. A diffusion model by Rau *et al*. (1996) predicts a hyperbolic relation between ε_p and CO_2. This means that ε_p is most sensitive to change in low pCO_2. The convexity of this relation decreases upon increasing μ. A higher sensitivity at low pCO_2 is also observed in the decreasing logarithmic relationship of ε_p vs. μ/pCO_2 (supplementary fig. 4; note: logarithmic x-axis). If *Microcystis* would rely on pure diffusive uptake of CO_2, a linear relationship between these parameters would be expected (Laws *et al*. 1995; Laws *et al*. 1997). The lack of a linear relationship here confirms that regulated carbon uptake occurs in our *Microcystis* strains (Burkhardt *et al*. 1999a). The dependence of ε_p on changes in active uptake is due to the fact that the activity of CCMs can affect both leakage and R_{HCO3}-. Induction of CCMs at low pCO_2 may therefore lead to changes in ε_p, promoting the sensitivity of ε_p to pCO_2. At high pCO_2 the activity of CCMs is decreased to a constitutive level (Price *et al*. 2008), where fractionation approaches its maximum value.

Another factor that clearly affects ε_p in our data is nitrogen limitation. In many different kinds of phytoplankton nitrogen limitation leads to increased ε_p (Hoins *et al*. 2016b; Hoins, 2016). It has been suggested that a higher ε_p under nitrogen limited conditions is due to changes in the energy balance in the cell, which potentially increases internal CO_2 recycling or active carbon uptake (Riebesell *et al*. 2000b, Hoins *et al*. 2016b; Hoins, 2016). The response of ε_p to pCO_2 in nitrogen limited conditions has been shown to strongly differ between/among species (Hoins *et al*. 2016b; Hoins, 2016). In nitrogen limitation some species fractionation even seem insensitive to changes in CO_2, such as the marine cyanobacteria *Trichodesmium* and the dinoflagellates *Alexandrium fundyense and Scrippsiella trochoidea* (Eichner *et al.* 2014; Hoins *et al.* 2016b; Hoins 2016). This is not the case with our data of *Microcystis*, which shows a higher sensitivity of ε_p to pCO_2 under nitrogen limitation than under nitrogen excess conditions.

The ε_p vs pCO_2 data were well described by logarithmic fits (fig. 5, supplementary table 1). A similar relation was found in field data of Smyntek *et al*. (2012). They modelled the relation based on algal physiology (Cassar *et al*. 2006) and supported this with data from a zooplankton species (*Daphnia galeata*) in a eutrophic lake. Just as in our results, a steep increase in ε_p occurs at low pCO_2, followed by a more or less linear relationship at medium to high pCO_2. In an extensive study of 16 different lakes by Morales-Williams *et al*. (2016), again a steep increase of fractionation is observed at low pCO_2, but no predictive relationship between ε_p and pCO_2 is found from medium to high pCO_2. These results suggest that ε_p may mainly be a clear indicator of pCO_2 at the low pCO_2 range. The effect of nitrogen limitation on the ε_p vs pCO_2 relation differs greatly between species, as was indicated in the previous paragraph. For *Microcystis*, nitrogen limitation has been shown to lead to a steeper relationship of ε_p vs CO_2, which increases the usefulness of ε_p as pCO_2 indicator.

Cellular leakage and bicarbonate usage

The response of leakage and R_{HCO3^-} to pCO_2 has been shown to vary considerably among different species of dinoflagellates (Hoins *et al*. 2015). While some species show a decrease of R_{HCO3^-} with increasing CO_2 and some species show an increase in leakage, others show no significant change in these parameters with increasing pCO_2 (Hoins *et al*. 2015). The proportion of bicarbonate to total fixed carbon can generally not be determined from fractionation data alone. For this, information about the cellular leakage is required (equation 3), which can be experimentally determined with Membrane Inlet Mass Spectrometry (MIMS). However, since shifts in fractionation values in this study are found to be very large in the nitrogen depleted treatment, ranging between -7.8‰ and 29.3‰, some estimates on the proportion of bicarbonate to total carbon uptake can be made. In these calculations, an intrinsic fractionation of RuBisCO was assumed to be 29‰, which is the intrinsic fractionation of the RuBisCO of spinach (Roeske and O'Leary, 1984). This value is close to the maximum observed fractionation by *Microcystis aeruginosa* in this study. Assuming this value, an increase in leakage can only explain a shift in fractionation up to 29‰. For all strains, the maximum shift in fractionation with increasing pCO_2 is larger: a maximum shift of 30.9-32.4‰ is shown. These values can be explained by a decrease of R_{HCO3^-} with increasing pCO_2, which means that the bicarbonate fraction of total carbon uptake is decreasing with increasing pCO_2, under nitrogen depleted conditions. Provided a shift in leakage explains a shift of fractionation of 29‰, the remaining 1.9-3.4‰ could indicate a decrease in R_{HCO3^-} of at least 0.20-0.36. Therefore, if a value of 29‰ for RuBisCO fractionation is a valid constraint and other uncertainties are <1.9‰, we can conclude that the proportion of bicarbonate to total carbon uptake decreases for all investigated strains with increasing pCO_2.

Using the extreme ε_p values allows further constraining of R_{HCO3^-} and leakage. Assuming minimal leakage, i.e. values approaching 0, eliminates the effect of ε_{rub} on ε_p, so at this point fractionation is completely dependent on R_{HCO3^-}. Fractionation values lower than 0 are therefore caused by bicarbonate uptake. Consequently, we can estimate minimum bicarbonate uptake. For determining maximum bicarbonate uptake, we set the upper constraint of leakage to a value of 1. We choose this value because: 1) the data show various ε_p values close to, and even slightly higher than ε_{rub}, 2) the lack of a reliable constraint on L_{max}, and 3) leakage is the only term in the model used to allow such high ε_p. With a maximum value of 1 for leakage in equation 3, the maximum proportion of bicarbonate to total carbon uptake can be calculated for the highest ε_p values. How these leakage constraints can be used for constraining R_{HCO3^-} in the applied one-compartment model (equation 3) is discussed in the next paragraph and is elucidated in figure 6.

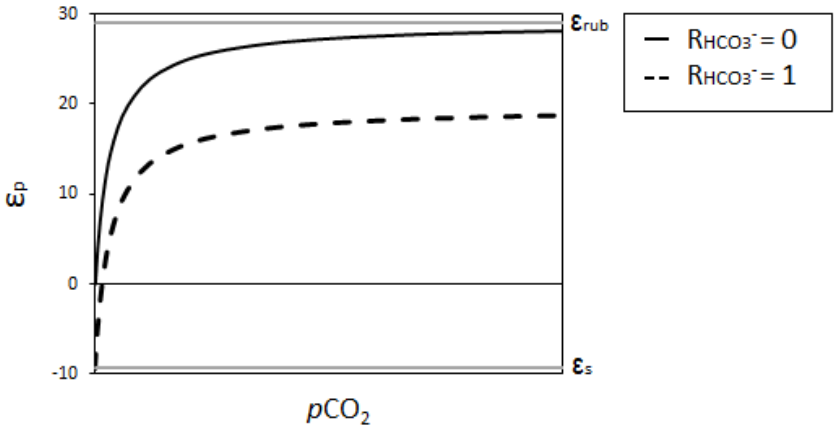


Figure 6. Relation between ε_p and pCO_2 using a one-compartment model. *Here is assumed that leakage increases with increasing pCO_2, while R_{HCO3^-} is set at to constant value. ε_p approaches the value of equilibrium discrimination of bicarbonate and dissolved CO_2 (ε_s = -9.3‰) at low pCO_2 (leakage approaching 0) if most carbon is taken up in the form of HCO_3^-, while ε_p approaches the intrinsic fractionation of RuBisCO (ε_{rub}= 29‰) at high pCO_2 (leakage approaching 1).*

The HUB-524 strain in the nitrogen depleted treatment has the broadest range of ε_p values. The negative ε_p values at low pCO_2 shown in fig.5 are caused by HCO_3^- uptake, since HCO_3^- is ^{13}C enriched compared to CO_2 and fractionation is expressed relative to CO_2. If we assume leakage to be 0 at these negative values, we can calculate the minimum fraction of bicarbonate to total carbon uptake by dividing the ε_p value by the value of equilibrium discrimination of bicarbonate and dissolved CO_2 (ε_s = -9.3‰, at 22°C; Mook *et al*. 1974) (fig. 6). Since the ε_p values at high pCO_2 under nitrogen limitation are very close to its maximum value (ε_{rub} = 29‰: RuBisCO fractionation), there is an upper limit to the possible contribution of R_{HCO3^-} to ε_p (since a higher contribution of R_{HCO3^-} decreases ε_p) (fig. 6). Therefore we can calculate the maximum fraction of bicarbonate to total carbon uptake value using leakage = 1 and again a value of equilibrium discrimination of bicarbonate and dissolved CO_2 (ε_s) of -9.3‰. The resulting calculations show that the minimum fraction of bicarbonate to total carbon uptake is 0.34 at low pCO_2 for the HUB-524 strain, while the maximum fraction at high pCO_2 is found to be 0 and 0.14 at the two highest pCO_2 treatments. This shows that the HUB-524 strain takes up a relatively large portion of HCO_3^- at low pCO_2, while at high pCO_2 this portion is much smaller.

In conclusion, the fractionation data indicate that all three *Microcystis* strains rely more strongly on CO_2 uptake at high pCO_2 than at low pCO_2. For the HUB-524 strain we concluded that it uses a relatively large proportion of HCO_3^- at low pCO_2, while it may be nearly completely reliant on CO_2 uptake at high pCO_2.

Fractionation models

If the proportion of bicarbonate uptake to total C uptake would have been measured, leakage could be calculated by using equation 3. Alternatively, it could be calculated from oxygen evolution and inorganic carbon fluxes measured by MIMS (see Badger *et al*. 1994, Rost *et al*. 2007, Kranz *et al*. 2009). Discrepancies between these methods have led to a reevaluation of the model used. Traditional models linking fractionation to leakage consider the cell as a single compartment, so that fractionation is directly dependent on the ratio between influx and efflux to and from the cell (fig.7A)

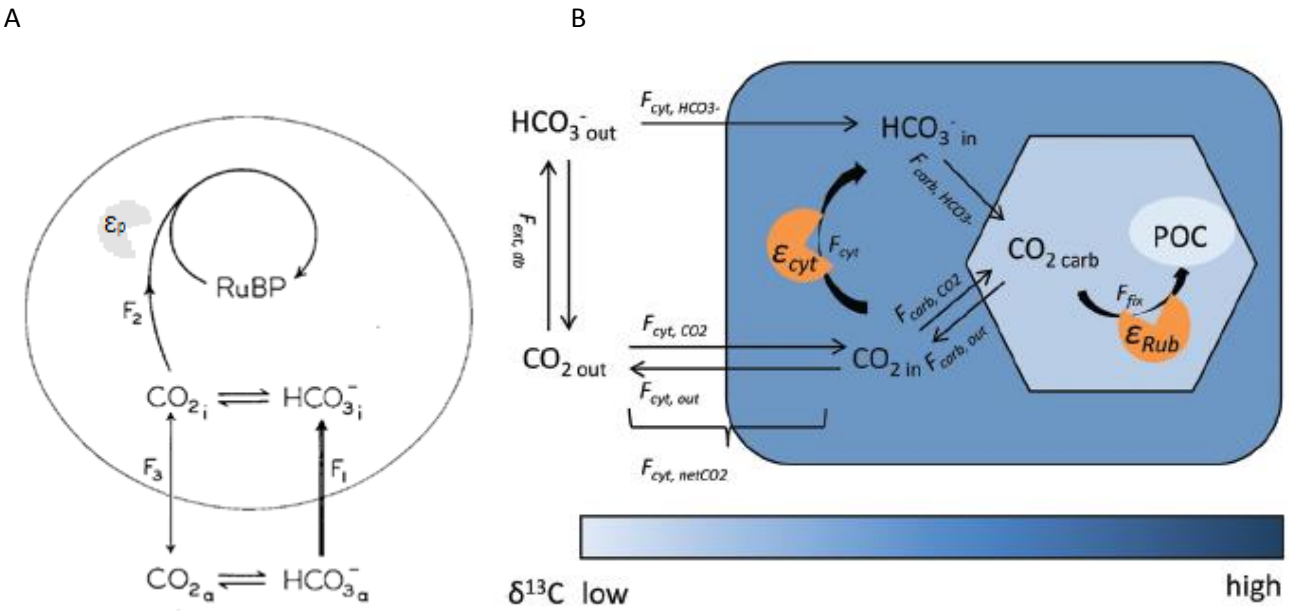


Figure 7. Fractionation models. *(A) Traditional one-compartment model (adapted from Sharkey and Berry, 1985 to include two carbon sources, as described in Burkhardt et al. 1999a). (B) Two-compartment model, by Eichner et al. 2015.*

A newer model for eukaryotic algae by Schulz *et al*. (2007) considers an additional compartment, the chloroplast, so that internal carbon cycling can occur in the cell. Internal carbon cycling can resupply lost carbon to the chloroplast, replenishing the ^{12}C pool used for fixation. Increased internal cycling may therefore increase fractionation, independent of leakage out of the cell. Leakage estimated from fractionation values using a traditional model, as is done in this study, may consequently be overestimated if internal cycling plays a significant role. This however does not allow maximum ε_p exceeding the intrinsic fractionation of RuBisCO. The ε_p values found in this study are much higher than the intrinsic fractionation found for the RuBisCO of cyanobacterium *Anacystis nidulans*. Though the RuBisCO in *Microcystis* may simply have a very high intrinsic fractionation, comparable to the RuBisCO of spinach, the observed values may also be explained by a two compartment model Eichner *et al*. (2015) (Fig. 7B) developed using the cyanobacterium *Trichodesmium*. The discrepancies for leakage estimation between the MIMS based approach and the carbon isotope approach is minimized if internal cycling not only resupplies lost carbon, but also fractionates. The internal cycling is described in figure 7B as F_{cyt}, with as fractionation ε_{cyt}, resulting in a lower $\delta^{13}C$ in the cytosol. In this research area, still much has to be resolved, but if indeed internal cycling is a process causing fractionation, it may be an alternative explanation for the high fractionation values found in this study.

Combined response of pCO_2 and nitrogen on carbon acquisition

Some distinct differences are shown in the ε_p data between nitrogen treatments. Firstly, ε_p seems to saturate at a much lower level in +N than in -N. Secondly, ε_p is more sensitive to a change in pCO_2 in –N (fig. 5). This indicates that the carbon uptake or leakage is strongly affected by the availability of nitrogen. In order to assess which factors influencing fractionation are affected by the nitrogen condition, we investigate the data in relation to the carbon fractionation model. According to equation 3, fractionation is a function of the proportion of bicarbonate to total carbon uptake of the cell (R_{HCO3}-) and of cellular leakage (L). R_{HCO3}- has been shown to be lower in *Microcystis aeruginosa* under -N conditions (Harke and Gobler, 2015), however, it is not known if and how this changes with increasing pCO_2. To our knowledge the combined effect of pCO_2 and nitrogen limitation on R_{HCO3}- in algae has not been investigated. The effect of increasing pCO_2 on R_{HCO3}- and L have been shown to vary considerably between dinoflagellate species (Eberlein *et al*. 2014; Hoins *et al*. 2016a). A strong preference for CO_2 as carbon source is connected with high values for leakage (Hoins *et al*. 2016a). The effect of bicarbonate on total fractionation is limited, as a maximum shift in R_{HCO3}- only leads to a shift in fractionation of 9.3‰ (ε_s; calculated from Mook *et al*. 1974). This effect is even likely to be lower, since the maximum R_{HCO3}- is typically less than 1, as shown by Verspagen *et al*. (2014b) who observed strongly impaired growth if cells were grown with bicarbonate as only carbon source. So, the effect of leakage on isotope fractionation is much larger than the effect of bicarbonate. Indeed, an increase in leakage with increasing pCO_2 explains the high increase in fractionation with pCO_2 observed in the nitrogen depleted treatment (-N). Leakage was previously described as a function of the carbon flux leaving the cell over the carbon flux entering the cell (equation 4). Alternatively, leakage (L) may be expressed into terms of demand and supply in the cell by rewriting equation 4 as:

$$L = 1 - \frac{C_{fixed}}{F_{in}} \qquad (5)$$

Where C_{fixed} is the carbon production per cell (demand) and F_{in} is the total influx of CO_2 (supply). Combining this with equation 3, gives the following expression for ε_p:

$$\varepsilon_{\mathrm{p}} = R_{HCO_3^-} . \varepsilon_{\mathrm{s}} + \varepsilon_{\mathrm{rub}} . (1 - \frac{C_{fixed}}{F_{in}}) \qquad (6)$$

In this expression, there are two unknowns (R_{HCO3}- and F_{in}). Either or both of these factors may be the cause of the differences in fractionation between nitrogen treatments. As previously indicated, in -N, leakage increases with pCO_2. This increase in leakage cannot be explained by the term C_{fixed} in equation 6, as only a slight change in C_{fixed} (carbon production) with increasing pCO_2 occurs and no consistent trend among strains, while ε_p and leakage do increase strongly in all strains. This means F_{in} is the major factor that increases with pCO_2 in -N conditions. So, despite a decreased uptake of bicarbonate at high pCO_2 (as was concluded earlier), total carbon influx increases with pCO_2 under nitrogen limitation.

Under low pCO_2, fractionation is higher in +N than in –N. This can be explained by either, a lower R_{HCO3}- in +N at low pCO_2, which can increase the value of ε_p, or by a higher leakage, which also increases ε_p. If we investigate a possible scenario in which we assume this effect is due to a higher leakage (so assuming no different response of R_{HCO3}- to pCO_2 in the different nitrogen treatments), the term $\frac{C_{fixed}}{F_{in}}$ in equation 6 must be lower in +N than in -N. A lower value for this term could be due to a lower carbon production rate in +N or due to a higher carbon influx. The data indicate that C_{fixed}

is much higher in +N than in -N at low pCO_2. This is in particular the case near 250 µatm pCO_2 where carbon production rates peak to a 7-fold higher rate in +N. Therefore, in this scenario, F_{in} must be higher in +N than in -N. Concluding, at low pCO_2, fractionation is higher in the nitrogen excess treatment due to a lower R_{HCO3^-} or due to a higher F_{in}.

ε_p shows in general a much stronger sensitivity to pCO_2 in -N than in +N. This difference in sensitivity may be due to a different response of R_{HCO3^-} or, alternatively, to a different response of leakage between the nitrogen treatments. ε_p may show a decreased sensitivity to pCO_2 if the R_{HCO3^-} decreases less with pCO_2 in +N. If we assume that the response of R_{HCO3^-} to pCO_2 is not different between nitrogen treatments, leakage would increase more strongly with pCO_2 in -N than in +N. Leakage, as defined in equation 5 is dependent on C_{fixed} and F_{in}. Since C_{fixed} shows little response to increasing pCO_2 in -N, while C_{fixed} decreases in +N, this indicates F_{in} increases more strongly in -N. In other words, total carbon uptake increases more strongly in nitrogen deplete conditions than in nitrogen excess conditions.

For nitrogen replete conditions, the situation is different. Since C_{fixed} in +N decreased with increasing pCO_2 from >250 µatm onwards, while leakage might increase (assuming no strong effect of HCO_3^-), it would mean that F_{in} increases less strongly in +N, remains constant or decreases with increasing pCO_2. Therefore, unless the difference can be explained by a different response of R_{HCO3^-} to pCO_2 between nitrogen treatments, total carbon uptake is affected differentially in nitrogen limitation.

Earlier, the putative decreased sbtA expression in high pCO_2 was discussed. Although this may decrease R_{HCO3^-} with increasing pCO_2, it is not known how this effect may be different between nitrogen treatments. A decreased sbtA expression may also affect F_{in}. This is because a decreased active bicarbonate transport might affect the total carbon uptake, if it is not balanced by increased passive or increased active CO_2 uptake. The negatively affected growth rates and carbon production rates in +N at high pCO_2 do suggest shifts in carbon uptake. Population densities in -N however do not seem affected (supplementary fig. 3). Possibly, population densities are not negatively affected by putative reduced sbtA expression because growth is already severely diminished because of nitrogen limitation.

A different response in F_{in} to pCO_2 in the two nitrogen treatments can indicate differences in active carbon transport (CCM activity) or passive diffusion (e.g. cell surface to volume ratio, membrane permeability). Cell volumes are indeed smaller in nitrogen deplete conditions, but a large difference is only observed for the NIES strain, and the PCC strain even has larger cell volumes in high pCO_2 (supplementary fig. 5), so this effect cannot explain a different response in F_{in} between nitrogen treatments for all strains. Membrane permeability data are unfortunately not available in this study, so further research is required to assess if passive or active transport is affected by pCO_2 differently under deplete and replete nitrogen conditions.

Limitations

This research was conducted with monocultures in controlled laboratory settings. A strong advantage of this method is that it allows us to investigate the fundamental changes that occur by modifying a single environmental factor. An obvious downside is that the high complexity of natural environments is not represented in these experimental conditions. This may make it difficult to directly translate these results to changes that occur in the field. Another factor that limits the translation of these results to the field is the imposed monoculture. In the field the existence of monocultures is extremely rare, if not non-existent. Competition with other phytoplankton and the grazing pressure of zooplankton will have a strong influence in the development of cyanobacterial populations. From this study it is therefore difficult to predict changes in natural environments. Despite the limited applicability for making predictions for natural environments, this study shows that changes in pCO_2 and nitrogen availability can lead to major changes on a cellular level and provides an important step towards understanding the fundamental responses of cyanobacteria to the combined effect of pCO_2 and nitrogen conditions.

Conclusions and outlook

An increase in pCO_2 has been shown to have large effects on *Microcystis* growth, stoichiometry and carbon acquisition. The direction and extent of these responses has been shown to be dependent on nitrogen conditions. Replete/deplete nitrogen conditions lead to an opposite response of C:N stoichiometry to increasing pCO_2, resulting in approximately doubled C:N ratios at high pCO_2 under N deplete compared to N replete conditions. The large changes observed in C:N stoichiometry indicate cyanobacteria are highly flexible and respond strongly to changes in the environment. Growth of *Microcystis* has been shown to be dependent on pCO_2, as growth is impaired at CO_2 limitation, while HCO_3^- is available in excess. Growth is also impaired at high pCO_2, which may be closely related to the carbon uptake systems present in the investigated genotypes, and by putative photoinbibition. Fractionation has been found to be more sensitive to increasing pCO_2 in nitrogen limitation. Here, the highest values for fractionation are found at high pCO_2. These values can be explained by a decreasing proportion of bicarbonate to total carbon uptake and an increasing leakage with increasing pCO_2. Furthermore, an increase in total carbon uptake has been shown to occur with increasing pCO_2 in nitrogen limitation. The extent of this shift may depend on nitrogen conditions. The relationship between ε_p and pCO_2 is strongest at low pCO_2 and in particular, under nitrogen limitation. Such strong relationship between ε_p and pCO_2 at low pCO_2 may be suitable for the development of CO_2 proxies. We show however that this relation will be complicated by the interactive effect of nitrogen conditions. Future atmospheric pCO_2 and climate change, affecting CO_2 concentrations in freshwater ecosystems may have major consequences for the growth, stoichiometry and carbon acquisition of cyanobacteria. Some of these changes may provide an ecological advantage to cyanobacteria, which could affect the phytoplankton community composition and possibly the development of harmful blooms.

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Supplementary information

Supplementary figures

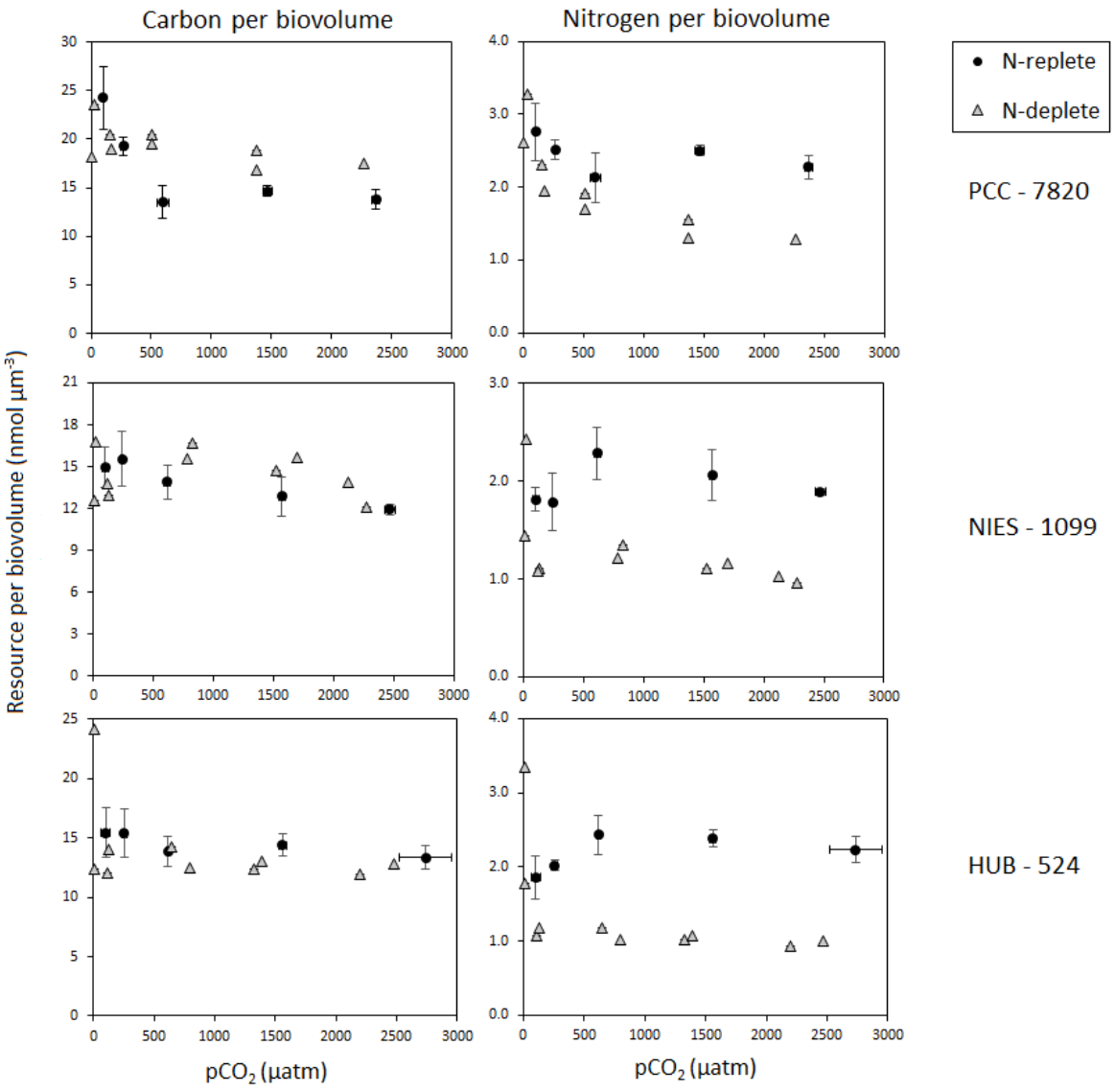

Figure S1. Carbon and nitrogen cell quota per biovolume. *For nitrogen replete treatment, 95% confidence intervals are shown (n=3). For nitrogen deplete treatment, all individual data points are shown (n=1).*

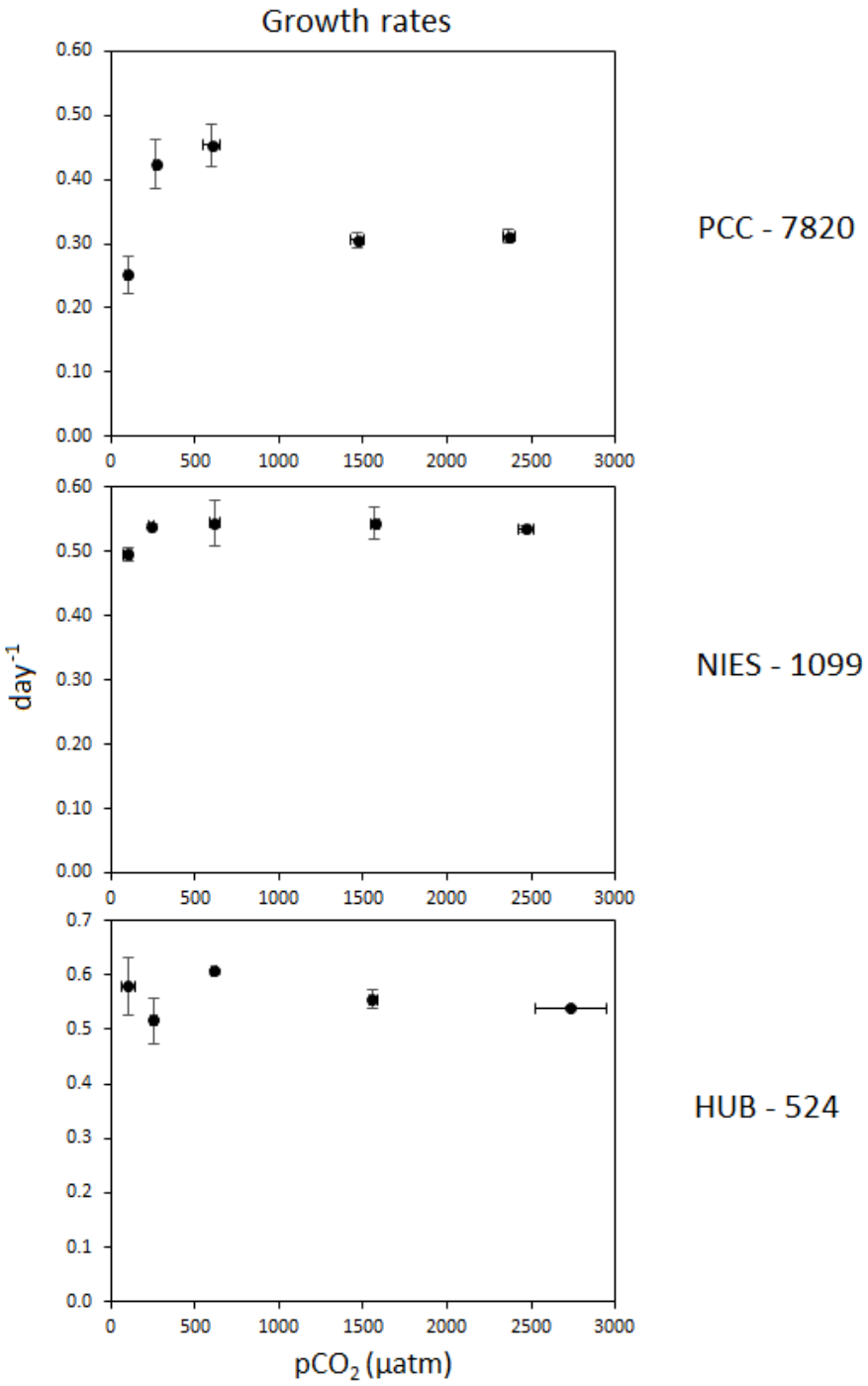

Figure S2. Growth rates. *These growth rates are determined in batch experiments, based on growth of number of cells. 95% confidence intervals are shown (n=3).*

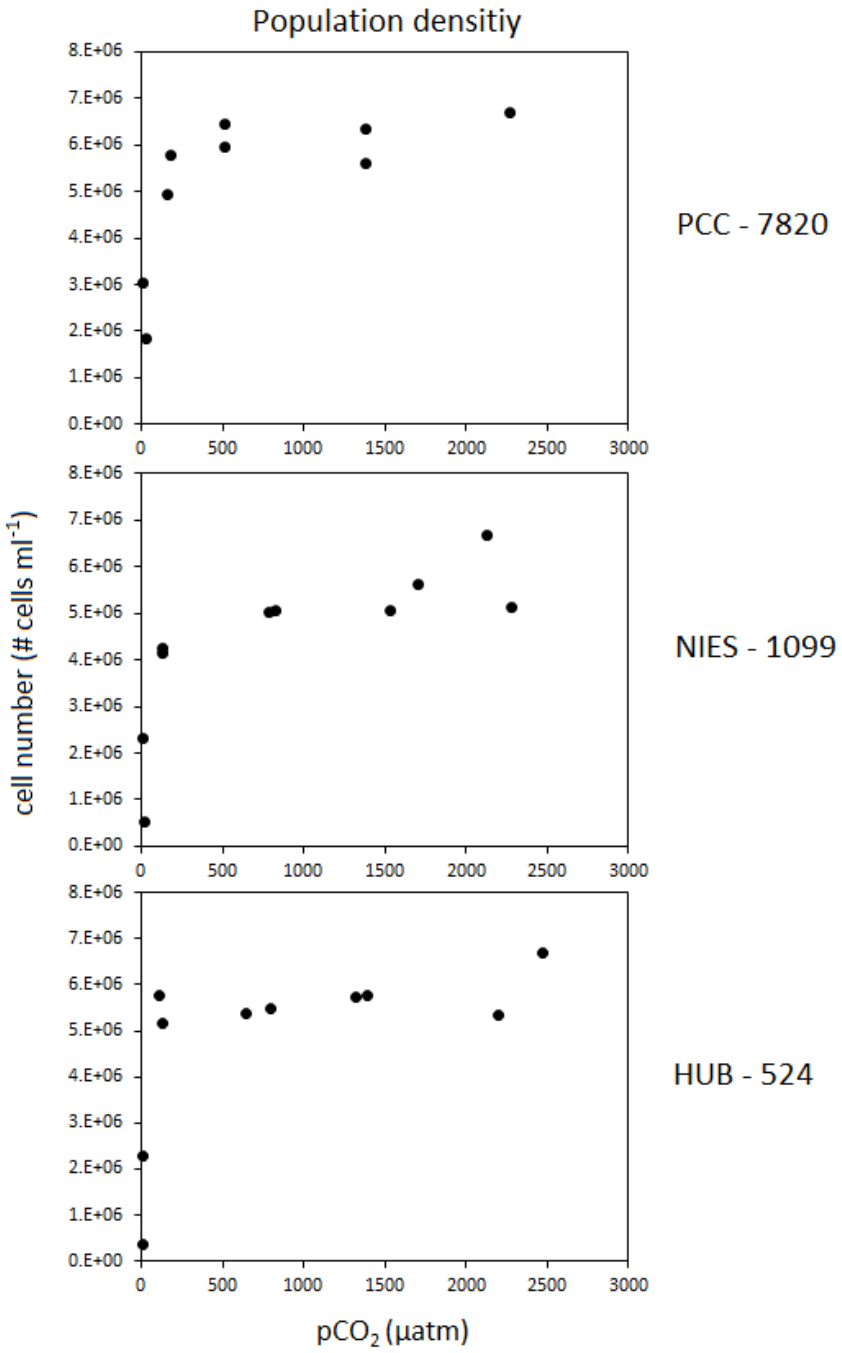

Figure S3. Population densities. *Populations on final day of each chemostat experiment are shown, after equilibrium was reached (n=1).*

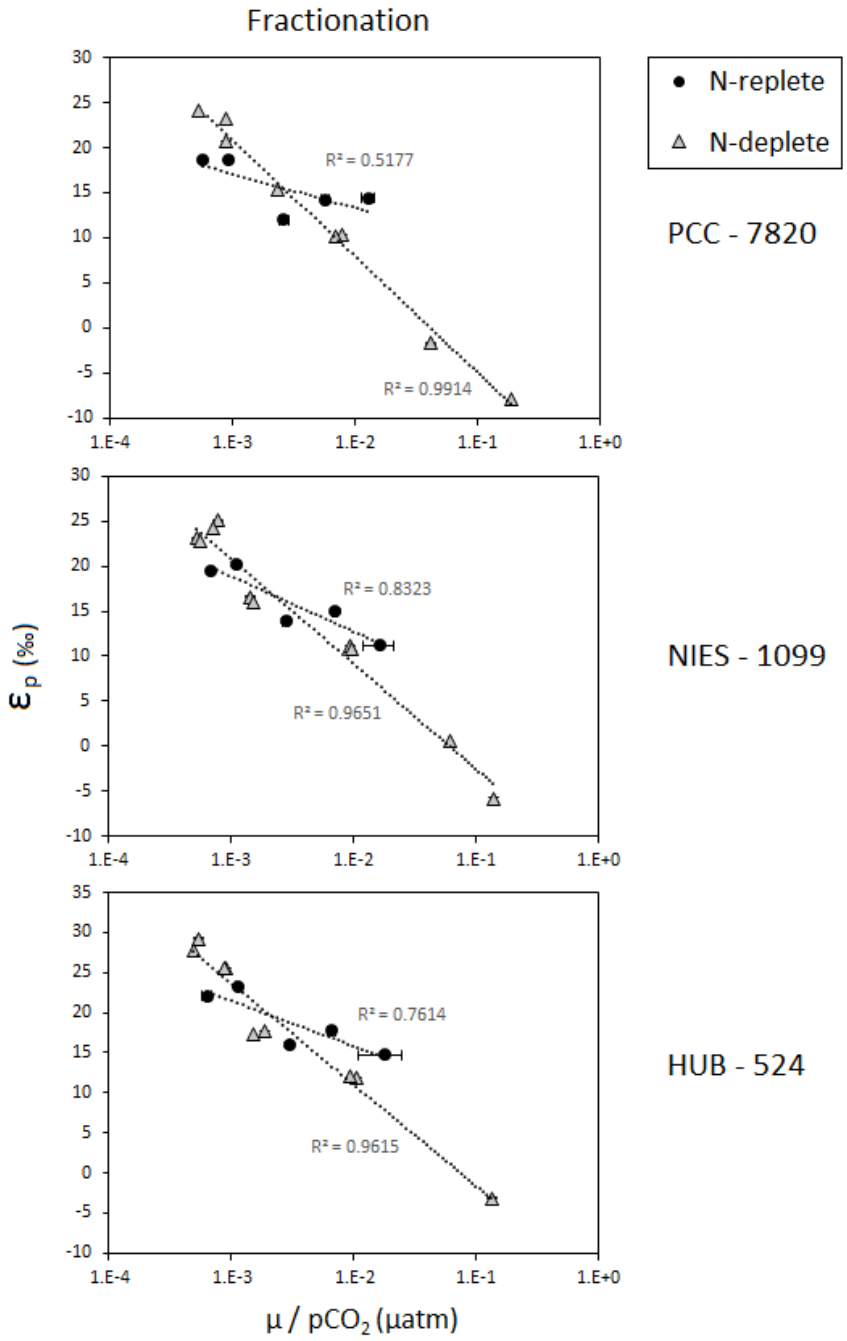

Figure S4. The relation of carbon isotope fractionation to growth rate / pCO_2. *This relation is described as a logarithmic function. For nitrogen replete treatment, 95% confidence intervals are shown (n=3). For nitrogen deplete treatment, all individual data points are shown (n=1). Trend lines are logarithmic best fits. The regression results are provided in supplementary table 3.*

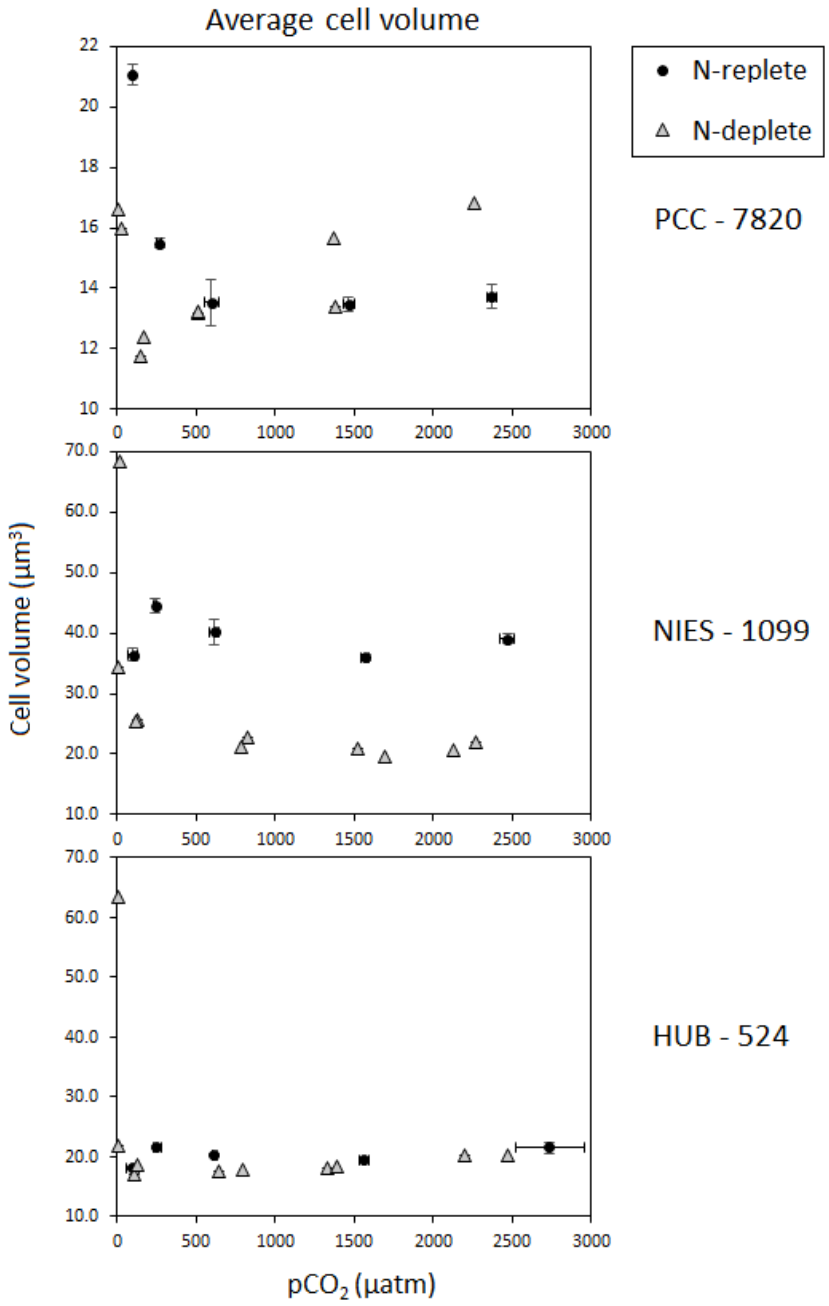

Figure S5. Average cell volumes. *For nitrogen replete treatment, 95% confidence intervals are shown (n=3). For nitrogen deplete treatment, all individual data points are shown (n=1).*

Supplementary tables

strain	Nitrogen replete	Nitrogen deplete
PCC – 7820	$1.548\ln(pCO_2) + 5.7729$	$5.606\ln(pCO_2) - 18.884$
NIES – 1099	$2.632\ln(pCO_2) - 0.7281$	$5.069\ln(pCO_2) - 15.105$
HUB – 524	$2.436\ln(pCO_2) + 3.2656$	$5.505\ln(pCO_2) - 15.352$

Table S1. ε_p vs pCO_2 regression lines

strain	Nitrogen replete	Nitrogen deplete
PCC – 7820	$-1.247\ln(\mu_c/pCO_2) + 4.8473$	$-5.416\ln(\mu_c/pCO_2) - 33.559$
NIES – 1099	$-2.446\ln(\mu_c/pCO_2) - 1.9326$	$-4.352\ln(\mu_c/pCO_2) - 22.222$
HUB – 524	$-2.406\ln(\mu_c/pCO_2) - 0.2025$	$-5.424\ln(\mu_c/pCO_2) - 31.322$

Table S2. ε_p vs Production rate/pCO_2 regression lines

strain	Nitrogen replete	Nitrogen deplete
PCC – 7820	$-1.644\ln(pCO_2) + 5.7413$	$-5.606\ln(pCO_2) - 17.862$
NIES – 1099	$-2.659\ln(pCO_2) + 0.5165$	$-5.069\ln(pCO_2) - 14.181$
HUB – 524	$-2.448\ln(pCO_2) + 4.5619$	$-5.505\ln(pCO_2) - 14.348$

Table S3. ε_p vs μ/pCO_2 regression lines